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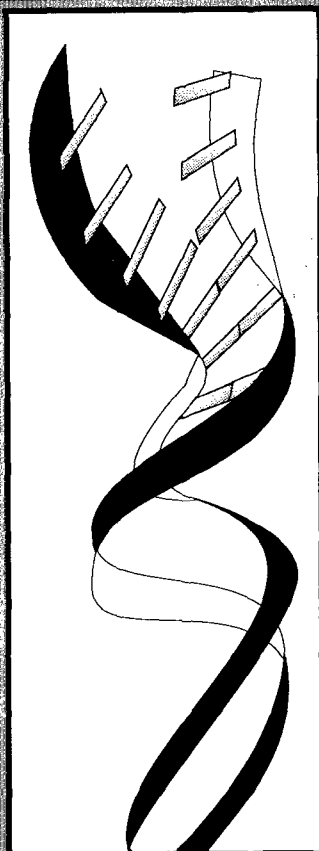
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SUBTYPES OF MUSCARINIC RECEPTORS

*Proceedings of the Seventh International Symposium
on Subtypes of Muscarinic Receptors*

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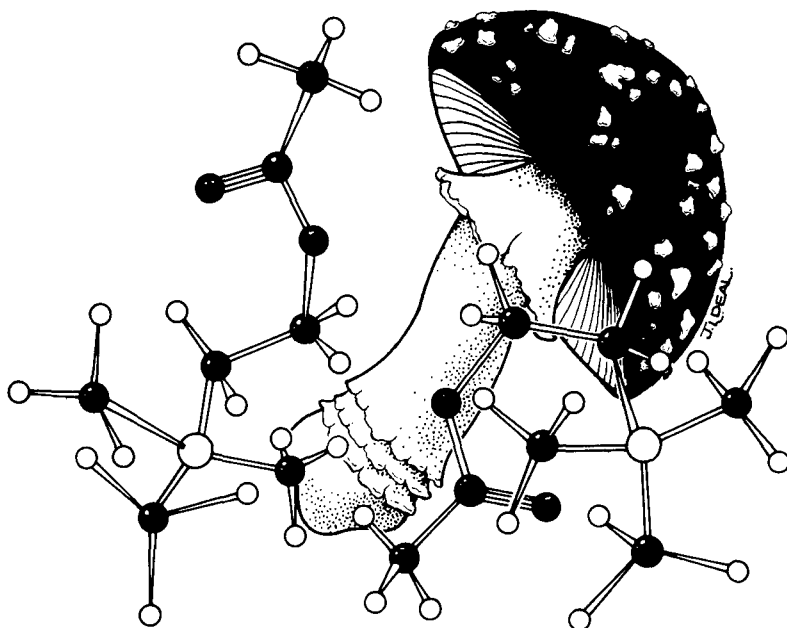
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SUBTYPES OF MUSCARINIC RECEPTORS

*Proceedings of the Seventh International Symposium
on Subtypes of Muscarinic Receptors*



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ROLE OF m1 RECEPTOR-G PROTEIN COUPLING IN CELL PROLIFERATION IN THE PROSTATE

Gary R. Luthin¹, Ping Wang¹, Honglin Zhou¹, Danny Dhanasekaran², and Michael R. Ruggieri³

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Summary

The prostate gland from several animal species contains variable levels of muscarinic subtypes, but only the human prostate expresses significant levels of the m1 subtype. We studied muscarinic receptor activity in human benign prostatic hypertrophy (BPH) as well as several cell lines derived from prostate cancer. The BPH we studied expresses approximately 75% of the m1 receptor and undetectable levels of the other receptor subtypes whereas PC3 cells express only the m3 receptor subtype. DU145 and LnCaP cells express approximately equal levels of m1 and m3 receptor subtypes. Only the PC3 cells responded to carbachol with an increase in turnover of polyphosphoinositides, and none of the cell lines responded with effects on cAMP metabolism. Co-precipitation of receptors with heterotrimeric guanine nucleotide-binding regulatory proteins demonstrated interactions of the m1 receptors with Gi, Gq and G16 in BPH tissue and of the m1 and m3 receptors with Gi, Gq and G12 in PC3 and DU145 cells. Mitogen activated protein kinase (ERK) activity was seen in response to carbachol in PC3 and DU145 but not LnCaP cells. Finally, carbachol promoted cell proliferation in all three cell lines. Thus, there appears to be no consistent relationship between ERK activity, cell proliferation, and the subtype mediating the proliferative response, amongst these prostate cancer cell lines.

Key Words: muscarinic receptor, prostate, G protein coupling, cell proliferation

Hammer and colleagues (1) were the first to use the drug pirenzepine to suggest that muscarinic receptors could be subdivided into at least two categories, then called M1 and M2. The M1 receptor was purified and subsequently cloned from mammalian cerebral cortex, a tissue rich in this pharmacologically defined receptor subtype (2). It is now well established that five receptor subtypes with differing protein sequences are expressed in brain. The m1 receptor protein (as defined by molecular sequence) is abundant in brain, constituting roughly 30-40% of the total muscarinic receptors present in several brain regions, yet is present in a mixture with other receptor subtypes. The pharmacological and biochemical analysis of this receptor subtype has thus focussed on the use of cell lines transfected with plasmids encoding and expressing the cloned m1 receptor DNA. We recently showed, using surgically-acquired samples of human benign prostate hypertrophy (BPH) and using an immunoprecipitation assay to measure expressed protein levels, that BPH expresses significant levels of the m1 receptor protein, constituting almost all of the measurable levels of muscarinic receptor in this gland (3). The role of the cholinergic innervation of this gland has

surprisingly not been well-characterized. We therefore chose this tissue as a model in which to study the m₁ muscarinic receptor in a prototypical physiological environment to relate the distribution, biochemistry and ultimately the physiological role of the m₁ receptor in regulating prostate function.

Methods

The methods used for immunoprecipitation of receptors and complexes of receptor and guanine nucleotide binding (G) protein have been described (3). The assays for phosphoinositide (PI) and cAMP metabolism were performed using whole-cell assays as described (4). Activity of mitogen activated protein kinase (ERK) was measured using a solid phase immunoassay (5) with myelin basic protein as a substrate for phosphorylation. Jun kinase (JUNK) was measured as described (5). [³H]Thymidine uptake into TCA-precipitable material was determined using conventional methods. Anti-G protein antibodies used in these studies were a generous gift of Dr. David Manning, University of Pennsylvania as described previously (3). PC3 and DU145 cells were a gift from Dr. Chung Lee, Northwestern University, and LNCaP cells were obtained from the ATCC collection.

Results

We have published that muscarinic receptors, measured using [³H]QNB binding, are present in prostate tissues from several species, at levels of 30-50 fmol/mg protein. Levels of the m₁-m₅ subtypes were different between species, and depended on the anatomical region (e.g., ventral, dorsal, lateral) investigated, which differs across species. To compare animal models to relevant human disease states, we measured levels of muscarinic receptor subtypes in BPH using samples derived from patients following transurethral radical prostatectomy (TURP) procedures (3). We found that in these samples, low to undetectable levels of muscarinic receptor subtypes of the m₂-m₅ class were found, but that roughly 75-80% of the detectable receptors were of the m₁ subtype, based on an immunoprecipitation assay using subtype selective antibodies. We have performed extensive studies to determine that those receptors that are refractory to precipitation are likely proteolysed, and cannot be measured in this assay (unpublished data).

We have since oriented our efforts to find a model system to study prostate function, using cell lines derived from normal and malignant prostate tissues. There are three widely used cell lines in which the cell biology has been extensively studied. The PC3, DU145 and LNCaP cell lines were derived from human prostate cancer cells. Unlike human prostate cancer, the PC3 and DU145 cell lines do not respond to androgen, whereas the LNCaP cells do. All of these cells have changed phenotype with passage, and therefore are not necessarily the ideal model with which to study prostate function and metastasis. However, they have given us systems to develop assays to measure parameters that would likely lead to fruitful examination of prostate function and the transformation into a malignant phenotype.

The PC3 cells express almost exclusively the m₃ subtype of receptor. The DU145 and LNCaP cells express roughly equal numbers of m₁ and m₃ receptors. Both the m₁ and m₃ receptors have been expressed in cells not normally known to contain these receptors, and have been shown under these conditions to actively promote metabolism of polyphosphoinositides (PI), and not to be active in the metabolism of cAMP. We used a whole-cell assay of PI and cAMP metabolism (4) to investigate this in the prostate-derived cell lines, expressing relatively low levels of receptor that are

comparable to those observed *in vivo*. In PC3, but not DU145 or LNCaP cells, carbachol promoted an increase in PI turnover that was reversed by atropine. This effect was insensitive to pertussis toxin, although all pertussis toxin substrates were inactivated. There was no effect of carbachol on cAMP metabolism in any of the cell lines. Therefore, and contrary to our expectations, the signaling of the m₁ and m₃ receptors was quite different in these cells, in particular regarding PI turnover.

We have developed an assay to measure interactions of receptors with G proteins. This relies on precipitation of the agonist-activated receptor with anti-receptor antibodies, followed by identification of G proteins that co-precipitate using western blotting. In BPH, this was shown to result in co-precipitation of Gi, Gq/11, and G16 alpha subunits with the m₁ muscarinic receptor (3). In PC3 and DU145 cells, the alpha subunit of G16 was not present, and in the presence of carbachol, precipitation of the m₁ (DU145) or the m₃ (PC3 and DU145) receptors resulted in co-precipitation of Gi, Gq, and G12 alpha subunits (submitted, Mol. Pharmacol). These findings were interesting, considering that expression of dominant positive ("gain of function") alpha subunits of G12 in NIH 3T3 fibroblasts induces cell proliferation and transformation, whereas expression of a dominant positive G16 construct inhibits these same effects. Some data similar to these were presented at the meeting and are reported *vide infra* (Gutkind *et al.*). This led us to pursue the effect of carbachol on two parameters associated with cellular proliferation, namely, the jun (JUNK) and mitogen activated (ERK) kinase pathways.

TABLE I. Precipitation of complexes of receptor and G protein from prostate cells.

<u>Tissue:</u>	<u>Subtype:</u>	<u>Gi</u>	<u>Go</u>	<u>Gq/11</u>	<u>G12</u>	<u>G13</u>	<u>G16</u>	<u>Gz</u>
BPH	m1	++	--	++	--	--	++	--
PC3	m3	++	--	++	++	++	--	--
DU145	m1	++	--	++	++	++	--	--
	m3	++	--	++	++	++	--	--

Legend: Specific anti-muscarinic receptor antibodies were used to precipitate complexes of agonist-bound receptor and associated G protein from BPH, PC3 and DU145 cells. G proteins were identified by western blotting as described (3).

In PC3 and DU145 cells, basal levels of ERK were elevated by incubation with carbachol, while basal levels of JUNK were unaffected. LNCaP cells do not exhibit either basal or stimulated levels of ERK. In all three cell lines, carbachol caused an increase in cellular proliferation. This occurred over a 2-4 hour time period in PC3 and DU145 cells in the presence, but not the absence of fetal calf serum. This was not inhibited by pertussis toxin under conditions where the pertussis toxin substrates were fully inactivated. These results have been compiled in a submitted manuscript.

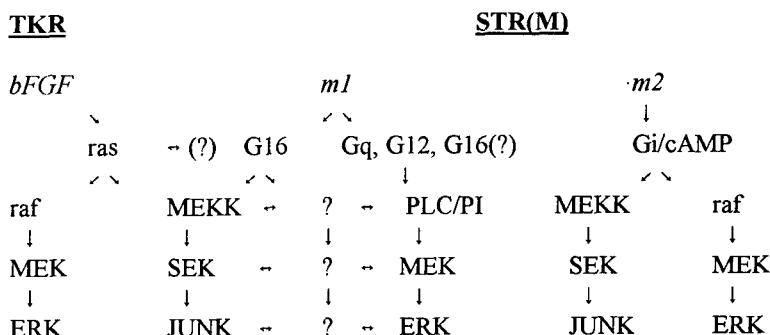
We used two cell lines derived from normal human prostate, obtained from the NIH (1532 and 1535), to compare with cancer-derived prostate cells using the above assays. Our results are preliminary, but suggest the following. These cells express low levels of the m₁ and m₃ subtypes of receptor (30-50 fmol/mg protein), yet neither cell line responds to carbachol with alterations in PI metabolism. We have shown consistently activation by carbachol of JUNK but not ERK in these

cells. Cell division was not changed in the presence of carbachol, measured using thymidine uptake.

Discussion

Our data show that the human prostate gland is unlike that of other animal species in that it expresses the m₁ muscarinic receptor exclusively in TURP samples, and a mixture of the m₁ and m₃ receptors in cell lines derived from normal and cancerous tissue. The levels of receptor in all cell types studied are virtually identical, ranging from 30-50 fmol/mg protein. These two receptor subtypes are thought to mediate PI turnover, yet only one cell line of five tested was shown to generate a PI response in the presence of carbachol. We measured this using a whole cell assay in the presence of lithium, and feel it unlikely (although possible) that our assay conditions prohibit measurement of this response. We have confirmed our ability to measure PI turnover in cells expressing cloned receptors (m₁ and m₃) in addition to the PC3 cells, and it is therefore not clear why this response was not seen in the other cell lines. We are currently using other measures of PI turnover that measure transient metabolite production to clarify this issue. As predicted from work using cells expressing recombinant receptors (6), no cAMP response has been detected in any of the cell lines used for these studies.

Figure 1. Hypothetical mechanisms for activation of ERK and JUNK through muscarinic receptors: comparison to mitogenic receptor signaling.



Legend: TKR, tyrosine kinase receptor; STR, seven transmembrane receptor; bFGF, basic fibroblast growth factor, an example of a mitogen; m₁, PI-linked muscarinic receptor; m₂, cAMP-inhibitory muscarinic receptor; ras, "small" mitogenic G protein; G16, Go, Gq, G12, PLC/PI-linked G proteins; Gi, cAMP-linked G protein. Other abbreviations are described in the text.

Both m₁ and m₃ receptors were found to co-precipitate with alpha subunits of Gi, Gq/11, and G12 in the cancer-derived cell lines, whereas in the TURP specimens we have published co-precipitation of the m₁ receptor with Gi, Gq/11 and G16 under the same conditions. This is interesting in light of findings using transfected NIH 3T3 cells, where dominant positive G12 constructs potently induced cell proliferation and transformation while similar G16 constructs inhibited cell proliferation (see 7 for review). By nature, the TURP specimens are not amenable to

study in the cell proliferation assay, so the relationship of m₁ receptor coupling to G16 in this tissue compared to G12 in cancer-derived cells is unclear. Our laboratory is attempting to derive cell lines directly from BPH specimens in primary culture to address some of these issues. We also wish to make the point that almost all previous studies on signaling pathways used by these receptors and G proteins have used cells transfected with cDNAs encoding cloned muscarinic receptor subtypes. By definition, these cells do not normally express muscarinic receptors, nor do they express significant levels of the respective G protein to be studied, and hence are used to reduce background "noise" that would potentially confound any results obtained after transfection with either receptor, G protein, or both. On the other hand, the cellular milieu in which these signal transduction events are studied cannot be considered in any sense normal in terms, particularly, of the expression level of receptor. The studies we present here rely on clonal cell lines, that likewise do not have phenotypes totally characteristic of normal, or for that matter, cancerous, prostate cells. Additionally, transfection with *predicted* GTPase-deficient, "gain of function" G protein mutants has not specifically been shown to render these proteins constitutively active in all reported usages.

While carbachol could induce elevation of ERK activity in PC3 and DU145 cells, though not LNCaP cells, all three cell lines were shown to respond to carbachol by an increase in cell number. In PC3 and DU145 cells (LNCaP were not studied) there was no effect of carbachol on JUNK activity. We point out that we do not measure levels of the respective kinase proteins in these assays, but rather the activity of the protein representing the phosphorylated and hence active form. In the cells derived from normal prostate, JUNK but not ERK activity was elevated in response to carbachol. The cancer-derived PC3, DU145, and LNCaP, and not the normal-derived 1532 and 1535 cell lines respond to carbachol by an increase in cell proliferation. This clouds the issue of the relationship between ERK, JUNK and cell proliferation in response to muscarinic receptor activation, at least in prostate cells in culture. Since all cell lines studied express identical levels of the same muscarinic receptor subtypes (m₁ and m₃), yet do not respond with the same second messenger response to receptor activation, we are unable at this time to pinpoint, even in a general way, the events that lead from receptor activation, to G protein coupling, and ultimately to cellular proliferation.

In summary, we are presenting what we think is a view of the m₁ (and m₃) muscarinic receptor proteins that varies greatly with published work regarding second messengers and predicted responses that have used transfected cell lines for these determinations. We have shown that these receptors couple as predicted to PI turnover in one cell line, yet not in another four that express the same level of receptor endogenously. We have shown dramatic effects of muscarinic receptor activation on cell division in cancer-derived cells, that is not seen in cells derived from normal prostate. However, the relationship between receptor activation and those signaling pathways thought to mediate cell division and transformation is, if anything, more complex than would be predicted based on assays using cells over expressing cloned receptors. We hope, through genetic approaches, to begin to unravel this web of nuance as our studies proceed.

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IN VIVO PHARMACOLOGY OF BUTYLTHIO[2.2.2] (LY297802 / NNC11-1053), AN ORALLY ACTING ANTINOCICEPTIVE MUSCARINIC AGONIST

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Summary

Butylthio[2.2.2] (LY297802 / NNC11-1053) is a mixed muscarinic cholinergic receptor agonist/antagonist that produces antinociception in mice and rats. As such, butylthio[2.2.2] may have therapeutic utility in the treatment of pain. Butylthio[2.2.2] was fully efficacious in the mouse grid shock, writhing, tail-flick and hot plate tests with ED₅₀ values ranging from 1.5 to 12.2 mg/kg after oral administration. In contrast, the ED₅₀ values for morphine ranged from 7.3 to 72 mg/kg after oral administration. Scopolamine was a competitive antagonist of the antinociceptive effects of butylthio[2.2.2]. Butylthio[2.2.2] did not produce either salivation or tremor at therapeutic doses; rather, there was a 50- to >100-fold separation between therapeutic doses and doses which produced side-effects. Butylthio[2.2.2] had high affinity for muscarinic receptors, but little if any affinity for other neurotransmitter receptors or uptake sites. In isolated tissues, butylthio[2.2.2] was an agonist with high affinity at M1 receptors in rabbit vas deferens, an antagonist at M2 receptors in guinea pig atria as well as an antagonist at M3 receptors in guinea pig urinary bladder. Although it has been suggested that M1 receptors mediate the antinociceptive effects of muscarinic agonists, M1 efficacy is not a requirement for antinociception, and, *in vivo*, the antinociceptive effects of muscarinic agonists are blocked by the intrathecal administration of pertussis toxin, indicating the involvement of m2 or m4 receptors. Since butylthio[2.2.2] is an M2 antagonist, antinociception is therefore most likely mediated by m4 receptors. Butylthio[2.2.2] is currently undergoing clinical development as a novel analgesic.

Key Words: butylthio[2.2.2], antinociception, M2 antagonist

Current drugs for the treatment of pain, including the treatment of acute and/or nociceptive pain, are less than ideal. For mild to moderate pain, the first line of therapy includes aspirin, acetaminophen/paracetamol, and nonsteroidal antiinflammatory drugs (NSAIDs). For

moderate to severe pain, NSAIDs still are the drugs of choice in Europe and Japan whereas opioid agonists and mixed agonist/antagonists also are used in the United States. Each of these classes of drugs has the potential to produce highly undesirable side-effects which limit their clinical utility. The currently available NSAIDs can produce gastrointestinal lesions and potentially fatal bleeding if used long enough at sufficiently high doses. Newer NSAIDs in development which selectively inhibit cyclooxygenase II (COX II) but not cyclooxygenase I (COX I) may avoid the gastrointestinal lesions of the nonselective compounds. However, there is a ceiling to the maximal pain relief which can be achieved with NSAIDs, and they are not very efficacious in relieving moderate to severe pain. Opioids are effective in relieving even severe pain, but the use of opioids is markedly constrained throughout the world because of their abuse liability and potential to produce physical dependence with repeated administration. Even when used only for short periods of time, opioids produce side effects such as respiratory depression, constipation and changes in sensorium which are problematic and limit their clinical use. Alternative pharmacologic approaches to the use of NSAIDs and opioids for the treatment of nociceptive pain are clearly needed.

A substantial body of literature, dating back approximately 50 years, has demonstrated that muscarinic cholinergic agonists as well as cholinesterase inhibitors produce antinociception in a variety of species and in a variety of tests for antinociception (e.g., 1-6). It is of interest to note that muscarinic agonists are as efficacious as full opioid agonists in more stringent antinociceptive tests such as the mouse tail-flick procedure (3,7), a test where weaker opioids are inactive. Moreover, the cholinesterase inhibitors physostigmine and tacrine have been reported to produce analgesia in humans as well as to potentiate the analgesic effects of morphine in humans (8-10). To date, no data have been reported on the potential analgesic effects of direct acting muscarinic agonists in humans, presumably because of the prominent parasympathomimetic effects of currently available muscarinic agonists. Taken together, however, these data suggest that the development of a muscarinic agonist which does not produce parasympathomimetic effects might provide clinically useful analgesia.

Parasympathomimetic effects such as bradycardia, hypotension, diarrhea, urination, salivation and lacrimation are most likely mediated primarily by peripheral M2 and M3 receptors. M2 receptors constitute approximately 90% of the muscarinic receptors in heart, and also are abundant in the ileum and peripheral lung (e.g., 11,12). M3 receptors are found predominantly in secretory glands and on smooth muscle such as ileum and bladder (11,13,14). M1 receptors also occur in the periphery in sympathetic ganglia, gastrointestinal tissue, and salivary glands (11,15,16) and thus may also contribute to some of the peripheral side-effects. Peripheral M4 receptors have been identified in rabbit peripheral lung, uterus and ileum (11,13); however, it is currently unknown what role, if any, these peripheral M4 receptors play in mediating undesirable parasympathomimetic effects. To date, M5 receptors have not been described in peripheral tissues. Thus, a muscarinic agonist devoid of activity at M2, M3 and possibly M1 receptors might be expected to be relatively free of peripheral parasympathomimetic effects.

A-delta and C-fibers which carry nociceptive information from nociceptors enter the spinal cord in the dorsal horn and synapse in Rexed's laminae I, II, III, IV and V, particularly in the substantia gelatinosa (lamina II). The second neurons in these pathways project via the spinothalamic tract, among others, to the ventrobasal nucleus of the thalamus. From the

thalamus, neurons convey nociceptive information to the cortex. Descending and centrifugal pathways are also important in modulating and carrying nociceptive information. Muscarinic receptors are abundant throughout these pathways. In the spinal cord, muscarinic receptors are present in laminae II and III and have been identified as being of the pharmacologic M1 and M2 subtypes (17). In the thalamus, the predominant muscarinic receptor is the m2 for which both mRNA (18) and protein (19) have been found. However, in addition to m2 receptors, protein for m1 and m4 (19) as well as mRNA for m1, m3, m4 and m5 (18) receptors have been found in the thalamus. In the cortex, m1 and m4 protein as well as mRNA are particularly abundant (13,18,19). Thus, muscarinic receptors are located throughout pain pathways, suggesting the possibility that muscarinic agonists could be acting at all levels of the central nervous system to modulate nociceptive information.

From extensive structure-activity relationship (SAR) studies (20), compound butylthio[2.2.2] (LY297802 / NNC11-1053; fig. 1) was chosen for development. The present report summarizes the pharmacology of butylthio[2.2.2].

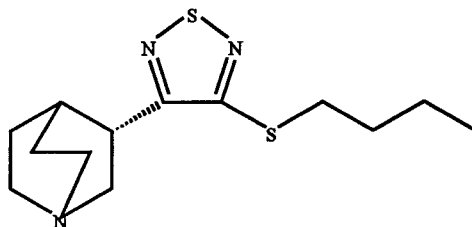


Figure 1. Chemical structure of butylthio[2.2.2]: (+)-3(S)-3-[4-butylthio-1,2,5-thiadiazol-3-yl]-1-azabicyclo[2.2.2]octane; LY297802 / NNC11-1053).

The antinociceptive profile of butylthio[2.2.2] was assessed in the mouse using the grid shock, tail-flick, hot plate and writhing tests. The ED₅₀ values for butylthio[2.2.2] ranged from approximately 0.2 to 1.5 mg/kg and 1.5 to 12.2 mg/kg after s.c. and p.o. administration, respectively, yielding p.o. to s.c. ratios ranging from 7 to 27 (table 1). In contrast the ED₅₀ values for producing salivation and tremor were >30 and 12 mg/kg s.c., and >60 and >60 mg/kg p.o., yielding therapeutic ratios of >130 and 54 s.c., and >40 and >40 p.o. Motor impairment or lethality were seen only at doses 116 and 254 times higher than the antinociceptive doses after p.o. and s.c. administration, respectively. In all antinociceptive tests, butylthio[2.2.2] was equieffective to and 3- to 24-fold more potent than morphine. The duration of action of butylthio[2.2.2] was similar to that of morphine. The muscarinic antagonist scopolamine was a competitive antagonist of butylthio[2.2.2] in that scopolamine produced dose-related shifts to the right in the dose-response curve for butylthio[2.2.2]. In contrast, the opioid antagonist naltrexone was without effect on the antinociceptive effects of butylthio[2.2.2]. The quaternary muscarinic antagonist metscopolamine did not antagonize butylthio[2.2.2] at doses equimolar to scopolamine, indicating that the analgesic effects were mediated centrally. After 6.5 days of repeated dosing in mice, there was marked tolerance to the antinociceptive effects of morphine, but minimal if any tolerance to butylthio[2.2.2]. Taken together, these data indicate that butylthio[2.2.2] is a potent and efficacious antinociceptive agent with a very favorable therapeutic window.

Table 1

Relative potencies for butylthio[2.2.2], oxotremorine and morphine in producing antinociceptive and side effects after s.c. administration in the mouse.

Test	Butylthio[2.2.2] <i>ED</i> ₅₀ (mg/kg)	Oxotremorine <i>ED</i> ₅₀ (mg/kg)	Morphine <i>ED</i> ₅₀ (mg/kg)
Writhing	0.19	0.05	0.69
Grid Shock	0.23	0.03	2.2
Tail-flick	0.39	0.05	9.2
Hot Plate	1.5	0.1	3.8
Salivation	>30	0.22	nt
Tremor	12.3	0.41	nt
Rotarod	27	0.07	nt

nt: not tested

In vitro, butylthio[2.2.2] had high affinity for muscarinic receptors in brain homogenates. The *IC*₅₀ values for inhibiting [³H]oxotremorine-M (agonist binding sites), [³H]pirenzepine (M1 receptors), and [³H]QNB (M2 receptors) were 0.32, 1.4 and 11 nM respectively. Butylthio[2.2.2] inhibited [3H]cytisine binding to nicotinic receptors with an *IC*₅₀ of 3120 nM. Butylthio[2.2.2] had little or no affinity for other neurotransmitter receptors and uptake sites. In isolated tissues, butylthio[2.2.2] was an agonist with high affinity for M1 receptors in the rabbit vas deferens, but was an antagonist at M2 receptors in guinea pig atria and at M3 receptors in guinea pig urinary bladder. These data indicate that, *in vitro*, butylthio[2.2.2] is highly selective for muscarinic receptors and is an agonist at pharmacologic M1 receptors but an antagonist at pharmacologic M2 and M3 receptors.

The pharmacology of butylthio[2.2.2] was further characterized *in vivo*. To assess activity at M2 receptors, the effects of butylthio[2.2.2] on heart rate and brain levels of acetylcholine were examined. Butylthio[2.2.2] produced only marginal increases in heart rate, but antagonized the heart rate-decreases produced by carbachol. Similarly, butylthio[2.2.2] was without effect on acetylcholine levels in rat brain. Taken together, these data indicate that butylthio[2.2.2] is a weak partial agonist at pharmacologic M2 receptors *in vivo* over the therapeutic dose-range. To assess the relative contribution of M3 receptors, the effects of butylthio[2.2.2] administered alone and in combination with oxotremorine in producing salivation were examined. Administered alone, the magnitude of the salivation produced by butylthio[2.2.2] was less than that of oxotremorine. Moreover, butylthio[2.2.2] decreased the salivation produced by oxotremorine to that of butylthio[2.2.2] alone. Thus, butylthio[2.2.2] appears to be a weak partial agonist at pharmacologic M3, as well as at M2, receptors *in vivo*.

Undesirable side-effects produced by opioids include constipation and respiratory depression (21). Butylthio[2.2.2] had no effect on charcoal meal transit in mice at doses which produced antinociception, while morphine produced the expected decrease in charcoal meal transit. Further, in guinea pigs, morphine produced dose-related decreases in ventilatory tidal volume, as expected. Butylthio[2.2.2] decreased tidal volume at lower doses, but at higher doses stimulated respiration. Taken together, these data suggest that butylthio[2.2.2] may have less liability to produce constipation and respiratory depression than morphine and thus may have an improved therapeutic profile over opioids.

It previously has been suggested that M1 receptors mediate the antinociceptive effects of muscarinic agonists (22,23). In order to ascertain the potential contribution of M1 receptors in producing antinociception, we compared the antinociceptive effects of butylthio[2.2.2] with two arecoline analogs (propoxy-TZTP and 3-Cl-propylthio-TZTP) which are relatively devoid of m1 agonist activity (Table 2) *in vitro*. Propoxy-TZTP and 3-Cl-propylthio-TZTP inhibit [³H]oxotremorine-M binding in rat brain homogenates with IC₅₀ values of 1.6 and 1.4 nM, respectively. As may be seen in Table 2, all 3 compounds produced antinociception, but none of the compounds were agonists at M1 receptors. Thus, M1 agonist activity is not required in producing antinociception.

Table 2

Comparison of the M1 efficacy and antinociceptive activity of butylthio[2.2.2], propoxy-TZTP and 3-Cl-propylthio-TZTP

Compound	Writhing	Grid Shock	m1 Pi ^a	
	ED ₅₀ mg/kg	ED ₅₀ mg/kg	Max %	EC ₅₀ μM
Butylthio[2.2.2]	0.19	0.23	18	>100
Propoxy-TZTP ^b	0.41	0.75	25	>100
3-Cl-Propylthio-TZTP ^b	2.0	0.84	5	>100

^a Inositol phosphate hydrolysis in BHK cells transfected with human m1 receptors;

^b TZTP: thiadiazole tetrahydropyridine

Muscarinic m2 and m4 receptors are coupled through pertussis toxin-sensitive G-proteins (Gi/o) to the inhibition of adenylate cyclase. We therefore administered pertussis toxin intrathecally (i.t.) to mice to determine if the inactivation of inhibitory G-proteins would block the antinociceptive effects of muscarinic agonists. In mice treated with vehicle, oxotremorine and butylthio[2.2.2] produced dose-related antinociceptive effects in the tail-flick test. In mice treated with 0.3 μg of pertussis toxin, the antinociceptive effects of oxotremorine were completely blocked (fig. 2). Similar results were obtained with butylthio[2.2.2]. The blockade of muscarinic antinociception by pertussis toxin demonstrates that m2 and/or m4 receptors mediate antinociception. Since butylthio[2.2.2] is an m2 receptor partial agonist with very low efficacy, these results suggest that antinociception is mediated by m4 receptors *in vivo*.

Discussion

Nonselective muscarinic agonists have long been known to produce antinociception in animals, but their clinical use has been precluded by their prominent parasympathomimetic effects such as salivation, tremor and diarrhea. The discovery of muscarinic receptor subtypes has spurred new interest in the development of compounds which might produce antinociception without parasympathomimetic effects by acting at one or a few muscarinic receptor subtypes. A focused drug discovery effort has led to the discovery of butylthio[2.2.2] which produces antinociception by actions at muscarinic receptors, but does not produce parasympathomimetic effects at efficacious doses, and has a wide therapeutic window.

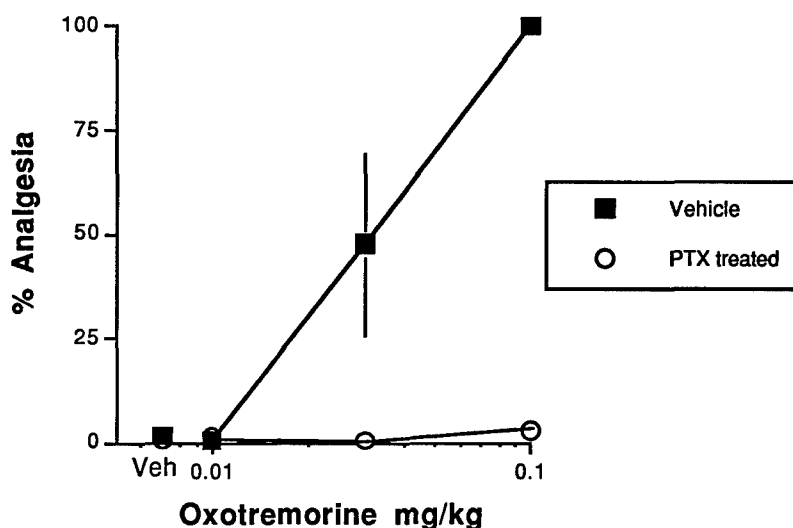


Figure 2. Dose-response curves for oxotremorine in the tail-flick test, using a 55 °C water bath, in mice treated intrathecally with either vehicle or 0.3 µg pertussis toxin 7 days previously. Abscissa: dose of oxotremorine administered s.c. in mg/kg. Ordinate: % maximum possible analgesic effect. Vertical lines represent \pm SEM and are absent when less than the size of the point. Points above Veh represent the effects of vehicle. Each point represents the mean of 5 mice.

Butylthio[2.2.2] showed efficacy in a wide range of antinociceptive tests, including writhing, grid shock, tail-flick and hot plate. NSAIDs, which are clinically useful in mild to moderate pain, are effective in the writhing test, but not in the more stringent grid shock, tail-flick and hot plate tests. Similarly, mixed agonist/antagonist opioids such as pentazocine and nalbuphine, which are clinically useful in the treatment of mild to moderate pain, are generally inactive in the hot plate and tail-flick tests. Only opioid agonists such as morphine which are clinically useful in treating even severe pain are efficacious in each of the writhing, grid shock, tail-flick and hot plate tests. Therefore, butylthio[2.2.2] might be expected to have more efficacy than NSAIDs or opioid mixed agonist/antagonists, and possibly as much efficacy as opioid agonists such as morphine in the treatment of nociceptive pain.

Butylthio[2.2.2] appears to have a wide therapeutic window relative to nonselective muscarinic agonists, NSAIDs and opioids. The therapeutic index for butylthio[2.2.2] for producing antinociception relative to salivation or tremor was 50- to 100-fold, respectively. Similarly, butylthio[2.2.2] produced minimal changes in heart rate over the antinociceptive dose range. The clinical utility of NSAIDs which inhibit both COX I and COX II is limited by their liability to produce gastrointestinal lesions, most likely as a direct consequence of COX I inhibition. In early toxicology studies, butylthio[2.2.2] did not produce gastrointestinal lesions and therefore is unlikely to produce such lesions in humans. Short term use of opioids is primarily limited by their propensity to produce tolerance, respiratory depression and constipation. In the present studies, the repeated administration of butylthio[2.2.2] produced little if any tolerance whereas the repeated administration of morphine produced the expected tolerance. Further, butylthio[2.2.2] did not produce respiratory depression at higher doses and had no effect on charcoal meal transit in contrast to morphine which depressed respiration and

decreased charcoal meal transit at doses which produced antinociception. Thus, butylthio[2.2.2], at analgesic doses, might be expected to be relatively devoid of the most problematic side-effects of NSAIDs and opioids.

Muscarinic receptors are abundant in pain pathways, including the substantia gelatinosa of the dorsal horn of the spinal cord, the ventrobasal nucleus of the thalamus, and the cortex. Thus, muscarinic agonists could modulate nociceptive information at any or all of these levels. The muscarinic receptor subtype involved *in vivo* has been previously suggested to be of the pharmacologic M1 subtype based on blockade by muscarinic antagonists. However, we identified two arecoline analogs, propoxy-TZTP and 3-Cl-propylthio-TZTP, which are relatively devoid of M1 agonist activity but were quite efficacious in tests of antinociception. The selectivity of M1 antagonists such as pirenzepine for cloned muscarinic receptors in CHO cells is only approximately 10- to 30-fold (24), suggesting that effects mediated by pharmacologic M1 receptors could be composed of actions at both genetically defined m1 and m4 receptors. Muscarinic m4, as well as m2, receptors are coupled to the pertussis toxin sensitive G-proteins Gi and Go. The intrathecal administration of pertussis toxin completely blocked the antinociceptive effects of systemically administered oxotremorine and butylthio[2.2.2] in the tail-flick test. Since butylthio[2.2.2] is a weak partial m2 agonist, the blockade by pertussis toxin suggests that the antinociceptive effects of muscarinic agonists such as butylthio[2.2.2] are mediated at least in part by m4 receptors in the spinal cord.

In summary, butylthio[2.2.2] is a novel muscarinic ligand which produces antinociception similar in magnitude to that produced by opioids at doses which do not produce parasympathomimetic effects. In addition, although further experiments are required, it appears that m4 receptors in the spinal cord play an important role in mediating the antinociceptive effects of muscarinic agonists. Butylthio[2.2.2] may thus have therapeutic utility in the treatment of pain as an alternative to NSAIDs and opioids.

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IN VIVO CONSEQUENCES OF M_1 -RECEPTOR ACTIVATION BY TALSACLIDINE

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Summary

The aim of this investigation in anaesthetized dogs was to provide direct evidence for an activation of the sympathetic nervous system by the muscarinic agonist talsaclidine (WAL 2014 FU). Intravenous infusion at a rate of 1 mg/kg/min increased plasma catecholamines and in particular epinephrine, thus indicating a predominant stimulation of the adrenals. Sympathetic activation was also indicated by increases in renal vascular resistance, an effect which was sensitive to α -adrenolysis. It is concluded that the sympathetic activation by talsaclidine is due to full agonism at the M_1 -receptor and the ability to cross the blood-brain barrier. As talsaclidine is less potent and only a partial agonist at M_2 - and M_3 -receptors many peripheral actions mediated by these receptor subtypes are functionally antagonized by the concomitant sympathetic activation.

Key Words: talsaclidine, Alzheimer's disease, sympathetic nervous system, adrenal medulla

As the memory impairment in patients with Alzheimer's disease seems to be causally related with the loss of cholinergic neurons, replacement therapy has been repeatedly attempted with inhibitors of acetylcholinesterase and muscarinic agonists. However, full clinical exploration of the compounds available has been hampered by intolerable side effects (see: 1). Talsaclidine (WAL 2014 FU) is a novel muscarinic agonist which promises fewer and less severe side effects because of its favourable receptor profile and pharmacokinetic properties (2, 3). This compound has been characterized in isolated tissues as a full agonist with higher potency at M_1 -receptors but as a partial agonist at M_2 - and M_3 -receptors.

In the anaesthetized guinea pig, intravenous injection of high doses of talsaclidine, in contrast to other muscarinic agonists such as arecoline, did not increase airway resistance (2). This discrepancy raised our interest and prompted us to investigate the mechanisms responsible. Muscarinic agonists contract the bronchotracheal smooth muscle by activation of M_3 -receptors and concomitantly induce liberation of catecholamines from sympathetic nerve endings and the adrenal medulla by activation of ganglionic M_1 -receptors (see: 4). These catecholamines give rise to β_2 -adrenoceptor-mediated functional antagonism of the bronchospastic effect.

TABLE I

Contractile Effects of Muscarinic Agonists in Isolated Tracheal Muscle from Guinea Pig. Shift of Concentration-Response Curves by 3 μ M Norepinephrine (NE) at the 5 % Level. Intrinsic Activity (IA) is expressed as Percentage of IA of Carbachol. Correlation of Ranking Orders (RO): Spearman Correlation Coefficient = - 0.79; $p = 0.0362$.

Compound	pD ₂	Log (EC ₀₅)	Log (EC ₀₅) +3 μ M NE	Difference	RO	IA	RO
Aceclidine	6.52	-7.91	-6.16	1.75	4	107	2
Arecoline	6.49	-7.45	-6.24	1.21	2	114	1
Carbachol	6.70	-7.50	-6.77	0.73	1	100	3
McN A-343	5.29	-6.63	-3.11	3.52	6	58	6
RS 86	6.66	-7.91	-6.22	1.69	3	90	4
Thiopilocarpine	5.30	-6.19	>-2.52	>3.67	7	36	7
Talsaclidine	5.17	-6.39	-2.93	3.46	5	63	5

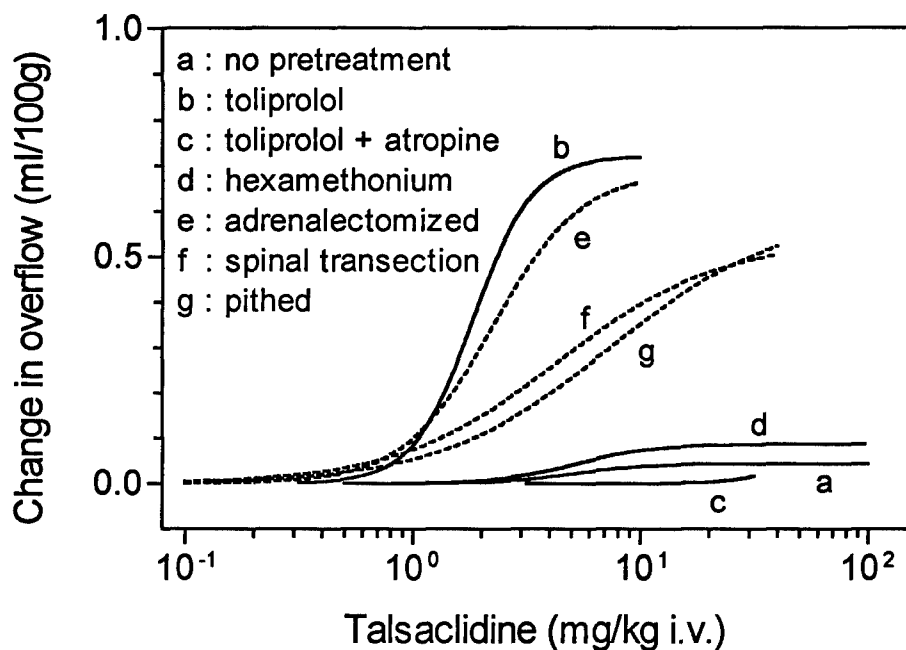


Fig. 1

Dose-response curves of the increase in bronchial resistance (change in overflow) in response to intravenous injection of talsaclidine into anaesthetized guinea pigs prepared according to Konzett and Rössler (5). The curves were calculated from medians derived from the results of individual curve fitting of the data from 5 - 6 animals. Toliprolol: 5 mg/kg i.v., atropine: 5 mg/kg i.v., hexamethonium: 0.2 mg/kg/min i.v.

In order to define the susceptibility to functional antagonism we therefore investigated talsaclidine and several other muscarinic agonists in isolated tracheal muscle. Evaluation of the concentration dependence of the contractile effect of seven muscarinic agonists in isolated tracheal muscle from the guinea pig revealed a wide range of intrinsic activities as well as susceptibilities to a functional antagonism by norepinephrine. The correlation between these parameters indicated that the resistance to functional antagonism by β -adrenoceptor activation increases with intrinsic activity (Table I). These data show that the bronchospastic effect of talsaclidine can be easily overcome by catecholamines (Walland and Hammer, submitted for publication).

Intravenous injections of talsaclidine to anaesthetized guinea pigs did not increase airway resistance over a wide dose range. However, the drug induced full bronchospasm in animals with β -adrenoceptor blockade or adrenalectomy (Figure 1). In guinea pigs with spinal transection or despinalization higher doses were needed for induction of bronchospasm and the maximal effects achievable appeared to be smaller (Figure 1). These results indicate that liberation of catecholamines from the adrenals in response to peripheral and central muscarinic receptor activation represents the essential broncholytic component of talsaclidine. On the other hand resistance to antagonism by catecholamines or inability to penetrate the blood-brain barrier seem to be responsible for the bronchospastic effects of the muscarinic agonists arecoline and McN-A-343, respectively (Walland et al., submitted for publication).

The present investigation in anaesthetized dogs was directed to providing direct evidence for the activation of the adrenal medulla by talsaclidine and the possible cardiovascular effects as a consequence of liberation of catecholamines.

Methods and Materials

Male and female beagle dogs and foxhound boxer mongrels with body weights from 10.1 - 33.5 kg were anaesthetized with intravenous pentobarbitone and were intubated for spontaneous respiration. A polyvinyl tube was introduced through a femoral vein and its opening was positioned in the vena cava at a site rostral to the renal veins for blood sampling. A Swan-Ganz® catheter was introduced through a brachial vein into the pulmonary artery for assessment of cardiac output by the thermodilution technique (6). Following median laparotomy electromagnetic flow probes were positioned on one or both renal arteries. A cannula was introduced into a femoral artery for blood pressure measurement. For registration and calculation of cardiovascular parameters a computer supported polygraph VAS-1 provided with equipment for thermodilution and electromagnetic flow measurement (IFD, Mühlheim, Germany) was used. Respiratory rate was evaluated from the CO₂-concentration with a respiratory gas monitor Normocap® 200 (Datex Instruments Corp. Helsinki, Finland).

Epinephrine, norepinephrine and dopamine were extracted from the blood plasma with heptane at pH 8.5. After extraction into phosphoric acid the catecholamines were separated by HPLC using an electrochemical detector for quantitative analysis. The assay and an enzymatic assay for glucose were performed by Biocontrol (Ingelheim, Germany). Talsaclidine was assayed after multiple extraction steps by gas chromatography-mass spectrometry. Talsaclidine, WAL 2014 FU, 1-Azabicyclo [2.2.2]octane,3-(2-propynyloxy)-, (R)-,(E)-2-butenedioate, was synthesized by Boehringer Ingelheim KG (Ingelheim, Germany).

Results

Intravenous infusion of talsaclidine:

Intravenous infusion of talsaclidine at a rate of 1 mg/kg/min into 5 dogs increased the rate of spontaneous respiration but eventually induced respiratory arrest after administration of a total of 46 to 58 mg/kg. As asphyxia affects adrenal secretion of catecholamines the results are only shown for the first 30 minutes of infusion, the time of maximum respiratory rate (Figure 2).

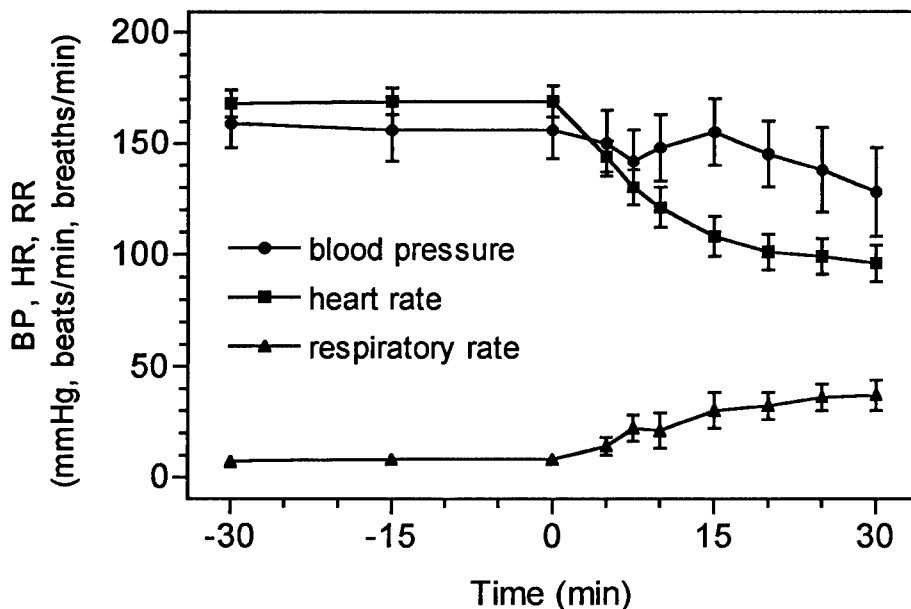


Fig. 2

Development of mean arterial blood pressure (BP), heart rate (HR) and respiratory rate (RR) in anaesthetized dogs during intravenous infusion of 1 mg/kg/min talsaclidine. Data points are means \pm SEM from 5 dogs.

Heart rate decreased continuously with infusion in all dogs whereas arterial hypotensive effects (Figure 2) and decreases in cardiac output were seen only in three animals. Total peripheral resistance remained essentially unchanged. The blood flow in both kidneys decreased during the infusion of talsaclidine. This was associated with an increase in renal vascular resistance which was maximum at 15 minutes (Figure 3).

During the control period plasma concentrations of dopamine, epinephrine and norepinephrine were at a low but stable level. During infusion of talsaclidine mean concentrations of norepinephrine and dopamine increased steadily and reached mean maxima of approximately 20-fold at 30 minutes. The epinephrine concentration increased dramatically: at 15 minutes a maximum level was reached which represented a >680-fold increase of the basal value. Hyperglycaemia developed steadily during the infusion of talsaclidine (Figure 4).

The plasma concentration of talsaclidine increased with infusion time, 7.1, 16.0 and 20.5 $\mu\text{g/kg}$ after 7.5, 15 and 30 minutes, respectively.

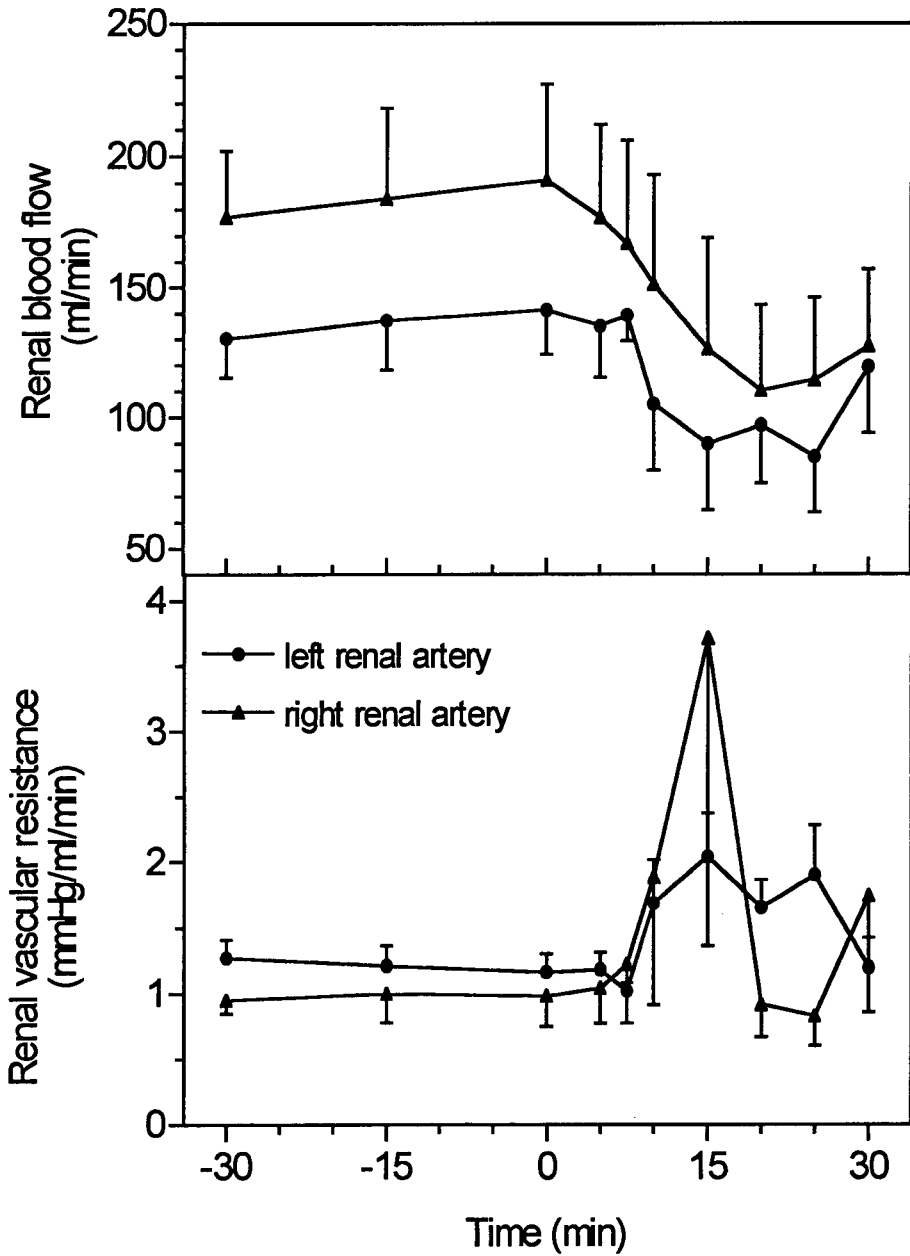


Fig. 3

Development of renal blood flow and vascular resistance during the experiments shown in Fig. 2.

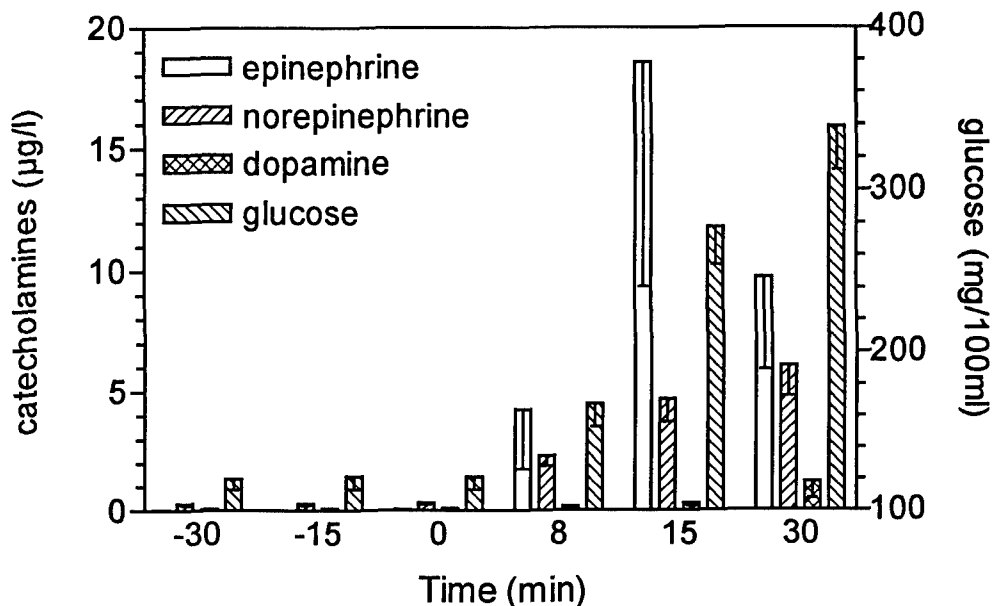


Fig. 4

Catecholamines and glucose in the blood plasma from the experiments shown Fig. 2.

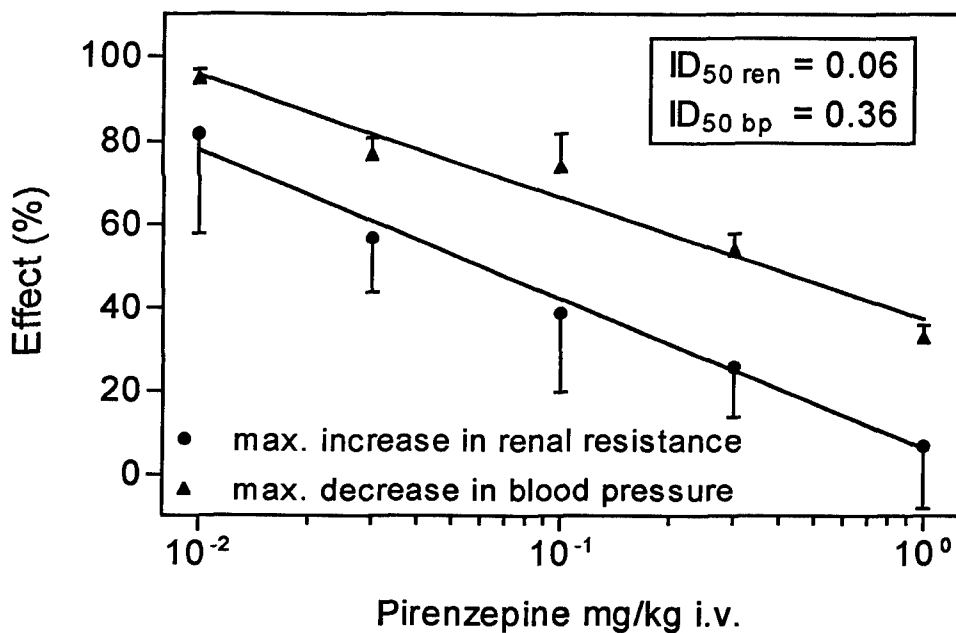


Fig. 5

Effects of repeated intravenous injections of 1 mg/kg talsaclidine during cumulative intravenous injections of pirenzepine in three anaesthetized dogs. The data are means \pm SEM of the maximal initial hypotensive effect and the maximal increase in renal resistance during the hypertensive phase, expressed as percentages of the effects before pirenzepine. Linear regression lines reveal correlation coefficients of - 0.9937 (renal resistance) and - 0.9776 (hypotensive effects). Both slopes are significantly different from zero ($P = 0.0006$ and 0.004).

Intravenous injection of talsaclidine

Intravenous injection of 1 mg/kg talsaclidine did not cause consistent changes in heart rate but exerted a biphasic blood pressure response. A short initial hypotensive phase was followed by a longer lasting phase of hypertension. Renal blood flow decreased and recovered only when blood pressure returned to control levels. The resulting increase in renal vascular resistance was most pronounced during maximal hypertension and was reproducible upon repeated injection of 1 mg/kg talsaclidine. Intravenous injection of 1 mg/kg talsaclidine into 3 dogs increased renal vascular resistance by $26 \pm 14.6\%$. Pretreatment with 5 mg/kg phentolamine i.v. reduced the effect of talsaclidine to $1 \pm 5.5\%$. In another group of 4 dogs, 1 mg/kg talsaclidine i.v. increased renal vascular resistance by $34 \pm 11.5\%$ but was ineffective ($0.3 \pm 2.4\%$) after intravenous injection of 5 mg/kg atropine.

The hypotensive effects and the increases in renal vascular resistance elicited by repeated injection of 1 mg/kg talsaclidine were dose-dependently inhibited by cumulative intravenous administration of 0.01 - 1 mg/kg pirenzepine, a M_1 -selective muscarinic antagonist. Pirenzepine was more potent as an inhibitor of the renal effect than of the hypotensive action of talsaclidine (Figure 5).

Discussion

The experiments with intravenous infusion of talsaclidine into anaesthetized dogs show that the plasma catecholamines increase in parallel with the blood levels of the drug. The plasma concentrations achieved in this study are in the range between 7.0 and 20.0 $\mu\text{g/ml}$. This is above the therapeutic plasma levels in patients which are expected to be well below 1 $\mu\text{g/ml}$. The increase in plasma catecholamines is a definite proof of an activation of the sympathetic nervous system by talsaclidine and confirms the conclusion drawn from guinea pig experiments (see: introduction). The elevation of the epinephrine concentration is particularly spectacular. In addition the ratio of epinephrine/norepinephrine concentrations increases from 0.1 during the control period to 3 after 15 minutes of drug infusion. These ratios are compatible with the catecholamine composition in canine tissues with sympathetic nerve endings, e.g. the heart, and the adrenal medulla, respectively (see: 7) and suggest that talsaclidine predominantly stimulates adrenal secretion. The results with talsaclidine in adrenalectomized guinea pigs (Figure 1) support this conclusion.

Hypoxia and hypoglycaemia also induce maximal discharge of the adrenal medulla (see: 8, 9) but are irrelevant in our experiments because talsaclidine increases the respiratory rate and induces hyperglycaemia, the latter effect resulting from adrenergic glycogenolysis.

Further evidence for the stimulation of the sympathetic system by talsaclidine is derived from some of its cardiovascular effects. The increase in renal vascular resistance in response to infusion or bolus injection of talsaclidine and the inhibition of this effect by phentolamine indicate the involvement of catecholamines and α -adrenoceptors. The complete prevention of renal vasoconstriction by atropine pretreatment shows that talsaclidine exerts its action indirectly via muscarinic receptors. The experiments with pirenzepine, a muscarinic antagonist with a preference for the M_1 -receptor (see: 4), indicate that the sympathetic activation by talsaclidine is mediated by M_1 -receptors while the initial hypotensive effect is due to agonism at M_2 - and M_3 -receptors (Figure 5).

In conclusion, the results available from this pharmacological study indicate that talsaclidine, as a consequence of its preference and full intrinsic activity at the M_1 -receptor, is a powerful activator of the sympathetic nervous system in general, and the adrenal medulla in particular. The ability of talsaclidine to access central muscarinic receptors may strengthen the sympathetic effects mediated by peripheral ganglionic M_1 -receptors. It is evident that the pronounced adrenergic component of action induces functional antagonism of various peripheral actions of the compound which are mediated by M_2 - and M_3 -receptors. The relative stability of the arterial blood pressure during infusion of talsaclidine may be a consequence of such an antagonism.

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MUSCARINIC M1 RECEPTOR AGONISTS INCREASE THE SECRETION OF THE AMYLOID PRECURSOR PROTEIN ECTODOMAIN

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Summary

Amyloid deposits in Alzheimer's disease are composed of amyloid β -peptides (A β) that are derived from the larger amyloid precursor protein (APP). Proteolytic APP processing is activity-dependent, and it can be regulated by muscarinic acetylcholine receptors. In particular, muscarinic m1 receptor subtypes increase cleavage within the A β domain, followed by the release of the soluble APP ectodomain (APPs). In this study, we show that the m1-selective agonist talsaclidine concentration-dependently increased APPs release from both transfected human astrocytoma cell lines and rat brain slices. This increase was blocked by atropine. In contrast, the M2 antagonist BIBN 99 failed to increase APPs release, and decreased it at higher concentrations. These results show that talsaclidine can effectively modulate α -secretase processing of APP in human cell lines and in brain tissue. The data suggest that talsaclidine may be a useful candidate drug to modulate APP processing in Alzheimer's disease.

Key Words: Alzheimer's disease, amyloid, secretase, talsaclidine, m1 receptor

The neuropathology of Alzheimer's disease (AD) is characterized by amyloid deposition in senile and diffuse plaques, as well as by neurofibrillary tangles. In addition, nerve cells degenerate, and the clinical manifestations of dementia in AD correlate best with the loss of both synapses and neurons (1). Cell loss is prominent in the cholinergic system but other major neurotransmitter systems are also heavily affected by the disease. The principal proteinaceous component of senile plaques is the β -amyloid peptide (A β) that is derived by proteolytic cleavage from the much larger amyloid β -protein precursor (APP). APP is a ubiquitous transmembrane glycoprotein with a single membrane spanning domain, a large N-terminal ectodomain and a short cytoplasmic C-terminal tail (2). Mature APP molecules are rapidly processed by various alternative proteolytic pathways, that generate soluble derivatives which are secreted into the extracellular space. The secreted ectodomain (APPs) results from proteolytic cleavage between residues 16 and 17 within the A β -domain by a suggested plasma membrane-associated endoprotease referred to as α -secretase (3). Alternatively, full-length APP can be reinternalized from the surface, and processed in an acidic intracellular compartment by cleavage at both the N- and C-termini of A β to yield A β molecules that are rapidly secreted (4,5). These peptides can aggregate and form amyloid. APPs has trophic and neuroprotective activities, and it was postulated that APPs plays an important role in the formation and the maintenance of synapses. APP knock-out mice, however, failed to demonstrate an obvious phenotype (6-9). In contrast, A β impairs cholinergic cell function, and it may be associated with neurotoxic and neurodegenerative processes that involve free radical generation, oxidation, destabilization, micro glial cell activation, and induction of apoptosis (10-14).

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Pathological changes in APP processing pathways are a central theme in genetic forms of AD: Disease-causing mutations of the APP or the presenilin genes either accelerate the generation of A β , or cause the formation of longer A β molecules that tend to aggregate more rapidly (15-18). The role of APP processing in sporadic AD is less obvious, but amyloid deposition is an early and invariant feature of all AD cases. Thus, treatment designs that aim at reducing amyloid formation may be effective in slowing the progression of the disease.

We have previously shown that the regulation of APP processing is activity-dependent: APPs release from brain slices is accelerated by increasing depolarization frequencies within the physiological range of pyramidal cell firing rates (19). Data from human brains seem to confirm that secretion of APPs is dependent upon cortical pyramidal cell activity, and on cholinergic function (20). Activity-dependent regulation of APP processing can be mediated by a large family of G protein-coupled cell surface receptors including muscarinic m1 receptor subtypes (21-23). Muscarinic m1 receptor activation stimulates α -secretase processing of APP in transfected cells, and in brain slices, and increased α -secretase processing is associated with decreased A β generation in most, but not all, cell types (24-29). m1 receptor subtypes are predominantly expressed in the central nervous system, whereas m2, m3, and m4 receptors are present in many peripheral tissues as well. Thus, subtype-selective m1 agonists can be expected to cause fewer peripheral effects, as compared to non-selective cholinomimetic compounds.

In this study, we tested the effects on APP processing of several muscarinic receptor ligands, including the m1 agonist talsaclidine (WAL 2014), both in transfected human astrocytoma lines and in freshly prepared tissue slices from the rat brain.

Materials and Methods

Brain slice preparation - The experimental protocol represents a modification of a previously reported procedure (24). In brief, three months old male Lewis B rats (Animal breeding center of the University of Hamburg) were used for talsaclidines experiments, and one year old male Wistar rats (Janvier, France) were used for BIBN 99 investigations. Animals were anesthetized with ketamine (200 mg/kg body wt.) and decapitated. The brains were rapidly removed into ice-cold Krebs-Ringer buffer (KRB) with reduced calcium (1.2 mM CaCl₂, 120 mM NaCl, 3.5 mM KCl, 1.2 mM MgSO₄, 1.2 mM NaH₂PO₄, 25 mM NaHCO₃, 10 mM glucose, pH 7.4, equilibrated with 95% O₂/5% CO₂), and with 1 mM ketamine (Sigma, Munich) (30-31). The hippocampi and striata were dissected from both hemispheres, and slices (300 μ m) were prepared at 4°C on a McIlwain tissue chopper (Bachofar, Germany). During a 80 minute equilibration period, 8-10 tissue slices in each chamber were continuously superfused with KRB at a constant flow rate (0.5 ml/minute; 37°C; without ketamine). A 95% O₂/ 5% CO₂ atmosphere was continuously maintained above each chamber to prevent hypoxia of the tissues. Four chambers were run in parallel for comparisons. During the following 60 minute time interval, 3 minute incubation periods were followed by 2 minute buffer-exchange periods (at 0.5 ml/minute flow rate). During these buffer exchange periods, 1 ml superfusate was sampled every 5 minutes for APPs assays. After 5 buffer exchange periods, Talsaclidine (0.01-1mM; Boehringer Ingelheim) or BIBN 99 (1 μ M - 100 μ M; Boehringer Ingelheim) was added to the superfusates. Physostigmine was used at 100 μ M. Atropine sulfate (100 nM - 100 μ M; RBI, Natick, MA) was applied either alone, or 10 minutes before exposition to talsaclidine as well as during talsaclidine exposition.

Cell culture - Clonal human U-373 astrocytoma cells stably transfected with muscarinic m1 receptors (density: 1182 fmol/mg protein) were stably co-transfected with human APP751. Cells were grown in 6 cm culture dishes, and the growth media were replaced by 1.5 ml serum-free

DMEM containing various concentrations of talsaclidine or carbachol. After 60 minute periods of incubation at 37°C, conditioned media were collected, centrifuged briefly to remove floating cells and debris, and supplemented with BSA at a final concentration of 10µg/ml.

APP analysis - Superfusate fluids from the brain slice preparation were collected on ice, centrifuged (14000 rpm, 4°C, 10 minutes), and 1.2 µl 2-mercaptoethanol (Serva) was added to each sample. 400 µl of each sample were absorbed on nitrocellulose membranes (BA-S85; Schleicher & Schuell) by using a vacuum-driven dot blotting device. The membrane was blocked in 1% casein, and incubated with a monoclonal antibody directed against the N-terminus of the APP ectodomain (clone 22C11; Boehringer Mannheim) overnight at room temperature. The blots were developed by using horseradish peroxidase-conjugated anti-mouse IgG (Amersham) as secondary antibody, and by enhanced chemiluminescence (ECL-detection reagent; Amersham) detected on linear x-ray films (DuPont). Blots were digitized with a CCD-camera (Enhanced Analysis System, E.S.A.Y. plus; Herolab), and quantitated by using the NIH-Image software (version 1.55; National Institutes of Health, USA). Secreted proteins in the conditioned cell culture media were precipitated with 200 µl 70% TCA, separated by SDS-PAGE, and transferred to a PVDF membrane. The blots were probed with the monoclonal antibody 6E10, and an ³⁵S-labeled secondary anti-mouse IgG antibody was used to detect bands by computer-assisted phosphor imaging.

Statistical analysis - Data were normalized to the average APPs release sampled after 95 and 100 minutes equilibration and expressed either as arbitrary units (AU) or as % control. Graphs represent means ± SEM. Groups were compared by two-tailed Student's *t*-test, and group differences were accepted as statistically significant at *p*<0.05.

Results and Discussion

Both carbachol and talsaclidine effectively increased α-secretase processing of APP in human U-373 astrocytoma cells stably transfected with human muscarinic m1 receptors within one hour of stimulation (Fig. 1). These data confirm that cells expressing m1 receptors couple α-secretase processing of APP to m1 receptor activity.

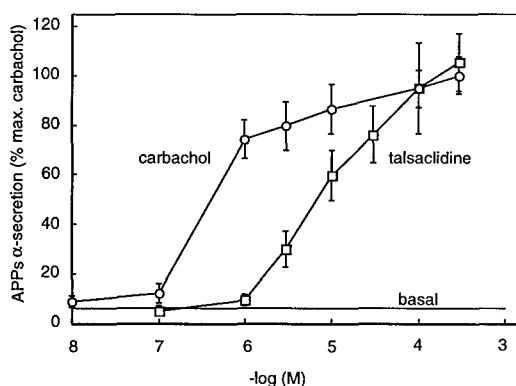


Fig. 1

Both the non-selective muscarinic agonist carbachol (circles) and the selective m1 receptors agonists talsaclidine (squares) stimulate α-secretase processing of APP in human astrocytoma U-373 astrocytoma cells stably expressing cloned m1 receptors. Data are means ± SEM from *n*=8 individual experiments.

In order to test whether this principle can be used pharmacologically to modulate APP processing in brain, we superfused freshly prepared tissue slices from the rat striatum, and followed the time course of APPs release in 5 minute intervals over a 30 minute period of drug exposure. Within this time period, talsaclidine consistently increased APPs release, whereas the basal APPs release from unstimulated control slices remained at a constant baseline or continued to drop slightly (Fig. 2).

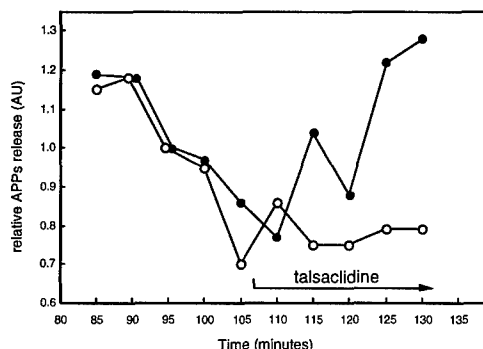


Fig. 2

Time course of relative APPs release from striatal slices. Filled symbols represent data from slices that were treated with talsaclidine after 107 minutes equilibration time. Open symbols represent the vehicle control. Data are from a representative experiment.

The increase in APPs release was concentration-dependent, and it was blocked by atropine, demonstrating that talsaclidine can regulate APP processing pathways in brain tissue via the activation of muscarinic receptors. Together with our previous report that muscarinic receptor-coupled regulation of APP processing is specifically mediated by m1 and m3, but not by m2 and m4 subtypes, these data strongly suggest that talsaclidine acts on APPs release in brain tissue via m1 receptors (Fig 3).

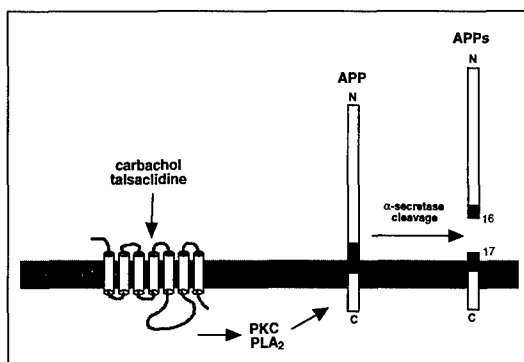


Fig. 3

Regulated APP processing. Talsaclidine increases α -secretase cleavage within the A β domain of APP. Receptor stimulation is coupled to APP processing by multiple signalling pathways including protein kinase C (PKC) and phospholipase A₂ (PLA₂).

Receptor-coupled increase in APPs secretion is mediated by stimulated α -secretase processing that cleaves APP within its A β -domain. As a consequence, A β formation decreases (25-28). In as much as brain concentrations of A β promote brain amyloid formation in AD, talsaclidine may be useful to slow amyloid formation (Fig. 3). Increased α -secretase-cleavage may be associated with

increased formation of p3, an APP derivative that comprises the 24 to 26 C-terminal residues of the A β domain (25). Because p3 is highly hydrophobic, and because it can aggregate *in vitro* it may be potentially amyloidogenic. There are several lines of evidence, however, that argue against a significant role of p3 in amyloidogenesis. The rodent sequence of p3 is identical to that of humans, yet rodents consistently fail to produce brain amyloid unless they express a human transgene (33, 34). The difference between rodent and human A β sequence consists of three amino-acid substitutions at positions 5, 10, and 13 within the N-terminus of A β , whereas the C-terminal sequences, specifically these of p3, are identical. The N-terminal amino-acid substitutions are believed to prevent amyloid formation in rodents. This is underscored by the histopathology of AD: Senile amyloid plaques are consistently detected by antibodies directed against the N-terminus of A β , and sequencing of senile plaque amyloid clearly identifies the presence of the A β -N-terminus and the absence of N-termini that start at residue 17. There is evidence for p3 in diffuse plaques, yet these structures are not associated with any appreciable signs of neurodegeneration (35). Furthermore, α -secretase processing in brain occurs normally throughout life, therefore p3 should be continuously produced, probably in an activity-dependent manner. Plaques composed of aggregated p3 peptides, however, are absent from normal brains. In conclusion, treatments designed to increase α -secretase processing could cause subtle increases in p3 that may well be irrelevant for both the progression and the pathophysiology of AD.

We next tested the effects of the M2 antagonist BIBN 99 on APP processing in hippocampal slices. Unstimulated APPs release also decreased continuously in the hippocampal preparation. BIBN 99 failed to increase APPs release, and higher concentrations decreased APPs release (Fig. 4).

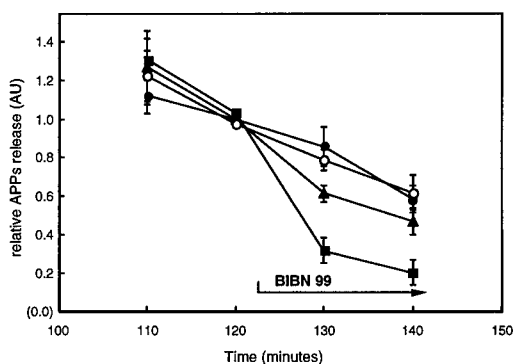


Fig. 4

BIBN 99 failed to increase APPs release from hippocampal slices. At higher concentrations, it decreased APPs release. Figure 4 shows the time course of mean APPs release from hippocampal slices. Data are means \pm SEM of $n=4-7$ individual experiments. Open circles: vehicle-treated control group, filled circles: 1 μ M BIBN 99, triangles: 10 μ M BIBN 99, squares 100 μ M BIBN 99. Mean APPs release after 9-19 minutes of treatment with 10 μ M and 100 μ M BIBN 99 were significantly ($p<0.05$) lower as compared to the vehicle control.

The observed decrease of APPs levels in brain slice superfusate samples was similar to that produced by the non-selective muscarinic antagonist atropine, suggesting that both drugs act via non-selective binding at the concentrations tested (Fig. 5). At the concentration used, physostigmine was ineffective in changing APPs release (Fig. 5).

Together with the observation that the non-selective muscarinic agonist carbachol failed to change APP processing in brain slices, our data strongly suggest that only selective activation of m1 subtypes, but not non-selective stimulation of muscarinic receptors effectively modulates APP processing in brain.

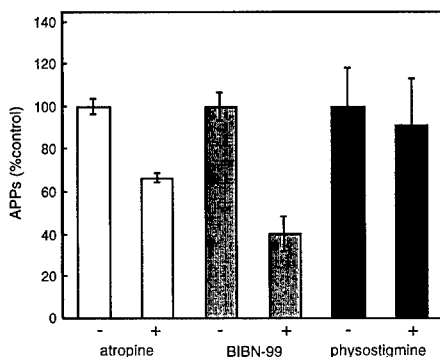


Fig. 5

Both the muscarinic antagonists atropine and BIBN 99 decreased APPs release but the choline esterase inhibitor physostigmine failed to change APPs release from brain slices. Atropine (100 μ M) was tested in striatal slices, BIBN 99 (100 μ M) and physostigmine (100 μ M). Data were normalized to basal APPs release values obtained 5 minutes before drug application. Bars represent means \pm SEM of $n=3-5$ individual experiments. Groups treated with either atropine or BIBN 99 were significantly different ($p<0.05$) from their respective controls.

Presented pharmacological investigations were performed using the superfused brain slice system with short incubation intervals. This technique is widely used for quantitative pharmacological analyses, and the results of our study show that it is also useful for the determination of secretory proteins.

In conclusion, talsaclidine effectively increases α -secretase cleavage of APP both in transfected human astrocytoma cell lines and in rat brain tissue. Studies in animal models are required to test whether it can reduce brain amyloid formation, and clinical studies are necessary to confirm that talsaclidine can modulate APP processing, and slow disease progression, in AD patients.

Acknowledgments

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MUSCARINIC AGONISTS IN ALZHEIMER'S DISEASE

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SUMMARY

As therapeutic agents, M1 agonists in the short-term may palliate symptoms of AD and improve memory function. In the long-term, M1 agonists have the potential to modify the underlying pathophysiology of AD, and thereby prevent or retard the course of dementia.

Key Words: muscarinic agonists, Alzheimer's disease, amyloid, memory

The rationale for considering muscarinic agonists in the treatment of Alzheimer's disease (AD) rests upon two themes: The cholinergic hypothesis of memory dysfunction, and muscarinic regulation of amyloid metabolism. The cholinergic hypothesis of memory dysfunction in AD stems from biochemical, pathological, and pharmacological evidence linking deficits in acetylcholine (ACh) to memory impairment. According to this hypothesis, administering muscarinic agonists to restore cholinergic tone would consequently improve memory in AD patients. Muscarinic regulation of amyloid metabolism is the second theme, and directs attention to treating the underlying pathophysiology of AD. Amyloid deposits in brain initiate a cascade of events leading to neuronal death. According to this line of reasoning, administering muscarinic agonists would prevent deposition of toxic amyloid fragments in brain tissue, and thereby retard progression of AD dementia. This chapter reviews these two complementary themes, and considers the future of muscarinic agonists for the treatment of AD.

THE CHOLINERGIC HYPOTHESIS OF MEMORY DYSFUNCTION IN AD.

The discovery of a cholinergic deficit in AD (1,2,3) ushered in the modern era of neuropharmacology in AD. In addition to confirming the selective decrease of choline acetyltransferase (CAT) activity in AD brain, subsequent investigations reported preservation of intact muscarinic receptor sites (4). These observations implied that drugs designed to enhance ACh synthesis or release, or to prevent its subsequent hydrolysis, might be beneficial in treating AD because the postsynaptic muscarinic targets were still intact.

Anatomic studies also supported the cholinergic hypothesis of memory dysfunction. The decrease in (CAT) activity in brains of AD patients correlated with estimates of dementia severity (5), and with neurofibrillary tangle counts in the medial temporal lobe (6). These observations indicated that neurons synthesizing ACh were severely damaged or dead, and focused research on the ventral forebrain CH1-4 neurons whose axons project to cortex and hippocampus (7). Whitehouse et al (8) subsequently reported severe atrophy and possibly

neuronal loss in these same ventral forebrain nuclei, especially the nucleus basalis of Mynert. Although atrophy of ventral forebrain nuclei is not unique to AD, finding degenerated neurons was internally consistent with biochemical indices of decreased cholinergic transmission in the terminal projections of their axons.

Pharmacological studies attesting to the importance of intact cholinergic neurotransmission in memory functions provided the third piece of evidence for the cholinergic hypothesis. It had been widely known that anticholinergic drugs impair memory and can even produce amnesia (9). Drachman and Leavitt (10) extended this knowledge and showed that low doses of scopolamine produced a pattern of cognitive deficits qualitatively similar to those observed in demented patients. Huff et al (11) refined this line of investigation and showed that low doses of scopolamine selectively impaired explicit memory without affecting other cognitive abilities. Subsequent studies reported that cholinergic agonists could enhance memory and learning whereas scopolamine had an adverse effect on these processes (12,13). Altered cholinergic neurotransmission may also contribute to non-cognitive neuropsychiatric aspects of AD (14).

These three lines of evidence provided a secure scientific basis for the cholinergic hypothesis of memory dysfunction in AD (15), which for the past 15 years has driven drug discovery and development programs in dementia. The implicit model has been Parkinson's disease, where there were numerous analogous findings: for example, in Parkinson's disease the loss of neurons in the substantia nigra pars compacta (in AD: nucleus basalis of Mynert) accounts for decreased production of dopamine (in AD: ACh) in the striatum (in AD: cerebral cortex and hippocampus) that results in extrapyramidal motor signs (in AD: amnesia). Administration of drugs such as levodopa to increase dopamine biosynthesis, or dopamine agonists to bypass the presynaptic deficit, improved the motor signs of Parkinson's disease. Could similar drug strategies work to palliate cognitive impairments in AD? Despite initial optimism, the results of clinical trials with cholinergic drugs in AD have shown disappointingly little benefit (Table 1).

Administration of choline and lecithin, the physiologic precursors for ACh biosynthesis, increased ACh levels in brains of animals (16,17) and in plasma and CSF of humans (18) but did not improve any aspects of cognition in AD. Benefits from acetylcholine esterase (AChE) inhibitors, given to prolong ACh synaptic activity, have been mixed. Physostigmine administration produced modest benefit in a few patients (19) but most investigators found that it exerted little or no long-term benefits. Most studies with physostigmine have been limited by its short duration of action and prominent side effects. A long-acting physostigmine compound is currently in clinical trials; it may obviate the side effects of regular physostigmine and enable higher and more prolonged increases in plasma levels. Tacrine also inhibits AChE, but it is a complicated molecule with many actions (20). Although tacrine has been approved by the FDA for the treatment of AD, numerous clinical trials attest to only a modest therapeutic benefit in a relatively small number of AD subjects (21,22,23). It is likely that other AChE inhibitors will be developed for treating AD. A preliminary report of one such drug, E2020, indicated benefits were in the same order of magnitude as those reported with tacrine (24). Despite the limited success in treating AD based upon neurotransmitter replacement with cholinergic drugs, the scientific basis for the cholinergic hypothesis of memory remains strong. Developing muscarinic agonists that are selective for M1 post-synaptic receptors in cerebral cortex and hippocampus have potential advantages over drugs that inhibit AChE as they may be more potent and have greater specificity, with fewer side effects.

TABLE 1

CHOLINERGIC DRUGS FOR TREATING AD

<u>DRUG CLASS</u>	<u>OUTCOME</u>
ACh PRECURSORS	
Choline	Ineffective
Lecithin	Ineffective
AChE-INHIBITORS	
Physostigmine	Ineffective, & Side Effects
Tacrine	FDA approved, Minimal Benefit & Substantial Side Effects
NON-SPECIFIC ACh AGONISTS	
Pilocarpine	Ineffective, & Side Effects
Arecholine	
Oxytremorine	
Bethanechol	
M1 MUSCARINIC AGONISTS	?

MUSCARINIC REGULATION OF AMYLOID METABOLISM

Amyloid formation is an early event in brains of AD patients, and defines much of the histopathology of AD. Amyloid is deposited in cerebral blood vessels as congophilic angiopathy, and within the neuropil as diffuse extracellular deposits, often with an associated neuritic reaction. Amyloid deposits in brain tissue consist primarily of aggregates of 40-43 residue peptides, termed A β . A β is derived by proteolytic cleavage of a transmembrane glycoprotein family known as amyloid precursor protein (APP). APP has a long extracytoplasmic N-terminal domain followed by a single transmembrane segment and a short intracytoplasmic C-terminal tail. The A β moiety begins 28 residues from the neuronal membrane in the extracytoplasmic portion, and extends 11 to 15 amino acids into the transmembrane portion. Under a variety of experimental conditions, the A β amyloid fragment deposited in AD brain is neurotoxic (25), whereas the N-terminal portion of APP may have neuroprotective and neurotrophic effects.

Based upon tissue culture data, several alternative proteolytic mechanisms have been identified that metabolize APP. There is an α -secretase pathway with cleavage within the A β domain itself that generates two non-amyloidogenic metabolites: a secreted N-terminal derivative and its cell-associated C-terminal counterpart. Endosomal-lysosomal APP processing generates multiple C-terminal derivatives, some of which contain intact A β sequences that are potentially

amyloidogenic. A β fragments can also be generated by other β secretase and secretase 2 pathways (26,27). Nitsch et al (28) established the first and most direct link between ACh transmission and APP processing when they showed that APPs secretion from HEK 293 cells separately transfected with the muscarinic m1 and m3 receptor subtypes increased within minutes after being stimulated by the muscarinic agonist carbachol. In subsequent experiments, they showed that stimulating the m1 receptor subtype with carbachol produced a concurrent decrease in A β secretion (29). Prior to these experiments, little was known about the regulation of APP processing, other than activation of protein kinase C with phorbol ester increased secretion of APPs from cells in culture (30). APP processing in wild-type HEK cells, as well as in cells expressing the cDNA for the m2 and m4 receptor subtypes, was not affected by muscarinic stimulation, indicating that APPs secretion was specifically linked to the m1 and m3 receptor subtypes. According to the working hypothesis, activation of α -secretase for APP processing depends upon the second messenger systems linked to protein kinase C, which is activated by receptor-coupled generation of diacylglycerol derived from phospholipid breakdown. This view explains why stimulating the m2 or m4 receptors did not influence APP processing, because their signaling pathways are coupled to cAMP mechanisms and not protein kinase C.

The regulation of biochemical pathways in the intact brain is much more complex than those isolated in cell culture model systems. Nonetheless, Nitsch et al (31) found that electrical depolarization of superfused rat hippocampal slices increased the release of endogenous ACh by 3-fold, and increased APPs release by 2-fold, within 50 minutes of stimulation. These findings demonstrated that action potentials can regulate APP processing in brain tissue, and encouraged trials to investigate whether APP processing can be modified by increasing ACh neurotransmission in the intact human brain. These studies have just begun in human subjects, and take advantage of the fact that APP fragments, including APPs and A β , can be detected in human cerebrospinal fluid (CSF) (32) and provide the closest, albeit indirect, measure of amyloid metabolism in the central nervous system. It is also known that mean CSF A β_{1-42} levels are decreased in AD compared to non-demented control subjects (33), and decreases in A β levels correlate with dementia severity (34). In our initial study with 10 AD patients, we tested long-acting physostigmine (kindly supplied by Forest Laboratories, NY) in doses between 9 and 15 mg /day. We measured APPs and A β levels in CSF before and then during drug administration, and found no significant drug change in either parameter. Lack of an effect on APP processing by physostigmine underscores the specificity of receptor subtype on APP processing: ACh combines with all receptor subtypes, but the effects on m2 and m4 may cancel the effects on m1 and m3 (35). With the development of muscarinic agonists that selectively activate m1 receptors, it will be possible to test the muscarinic-amyloid hypothesis more directly than in the past. Future studies will determine whether these new agonists decrease A β levels in CSF of AD patients, or reduce the A β burden in brains of transgenic mice that produce amyloid plaques (36,37). If successful, these results would inaugurate a new era in neuropharmacology: For the first time it would be possible to attack fundamental mechanisms leading to neuronal degeneration and AD dementia.

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THE PATHWAY CONNECTING m2 RECEPTORS TO THE NUCLEUS INVOLVES SMALL GTP-BINDING PROTEINS ACTING ON DIVERGENT MAP KINASE CASCADES

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Summary

m1 and m2 receptors are traditionally linked to tissue specific functions performed by fully differentiated cells. However, these receptors have been also implicated in growth stimulation. The mechanisms whereby these receptors regulate proliferative signaling pathways are still poorly understood. Furthermore, pharmacological evidence suggest that many growth promoting agents act on G_i coupled receptors, but there is no formal proof that induction of DNA-synthesis results from decreased intracellular levels of cAMP. In our laboratory, we have used the expression of m1 and m2 receptors as a model for studying proliferative signaling through G protein-coupled receptors. Currently available evidence suggest that these receptors signal to distinct members of the MAP kinase superfamily, MAP kinase and JNK, through $\beta\gamma$ subunits of heterotrimeric G proteins acting, respectively, on a Ras and Rac1 dependent pathway.

Key Words: G proteins, muscarinic receptors, MAP kinase, JNK, Ras, signal transduction

In most cell types, the stimulation of protein tyrosine kinase receptors or G protein-coupled receptors results in the elevation of the enzymatic activity of extracellular signal-regulated kinases (ERKs), also termed MAP kinases, and this family of serine-threonine kinases is believed to be critical for the transduction of proliferative signals (1). The stimulation of tyrosine kinase receptors provokes the activation of MAP kinase in a multistep process. For example, essential molecules linking EGF receptors to MAP kinase include the adaptor protein GRB2/SEM-5, a guanine nucleotide exchange protein such as SOS, p21^{Ras}, and a cascade of protein kinases defined sequentially as MAP kinase kinase kinase, represented by c-Raf-1, and MAP kinase kinase such as MEK1 and MEK2 (2). MEKs ultimately phosphorylate p44^{MAPK} and p42^{MAPK}, also known as ERK1 and ERK2, respectively, in both threonine and tyrosine residues thereby increasing their enzymatic activity. In turn, MAP kinases phosphorylate and regulate the activity of key enzymes and nuclear proteins which then regulate the expression of genes essential for proliferation (1). The mechanism(s) of MAP kinase activation by receptors acting through G proteins is still poorly understood (3,4). Because of the proposed central role of this family of kinases in linking cell surface receptors to the nucleus, we set out to investigate the pathway connecting G protein-coupled receptors to MAP kinases.

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Signaling from G protein-coupled receptors to MAP kinase involves $\beta\gamma$ subunits of heterotrimeric G proteins acting on a Ras-dependent pathway

To study the mechanism of activation of MAP kinases by G protein coupled receptors we used the expression of an epitope tagged-MAP kinase together with m1 or m2 receptors in Cos-7 cells. The advantage of this system is that a number of proteins can be simultaneously expressed at high levels, without the influence of biological and biochemical changes that might occur during prolonged culturing of stable transfected cells. We observed that in cells expressing either muscarinic receptor carbachol induced an increase in HA-ERK2 activity in a concentration dependent manner (Fig. 1) (5). Thus, ERK2 is activated by both m1 and m2 receptors in these cells.

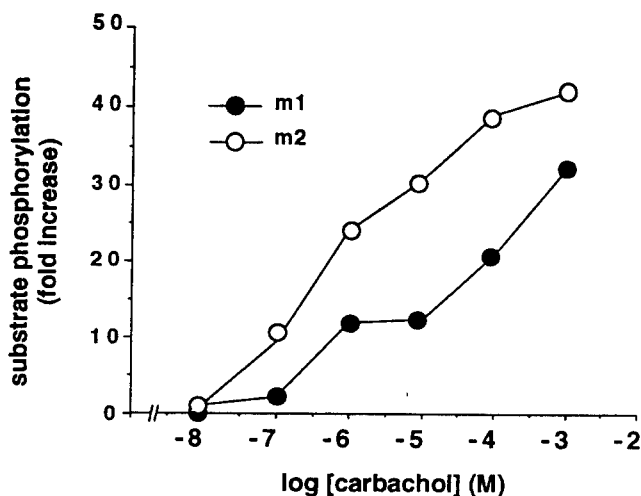


Fig. 1

Carbachol increases the enzymatic activity of an epitope-tagged ERK2 when coexpressed with m1 or m2 receptors in Cos-7 cells. Cos-7 cells were transfected with an expression plasmid for the epitope-tagged ERK2 and m1 or m2 receptors, serum starved overnight, lysed upon stimulation with the indicated concentrations of carbachol for 5 min, and ERK kinase activity was determined in anti-HA immunoprecipitates as described (5). Data are represented as fold increase with respect to unstimulated cells.

As expected, treatment of transfected Cos-7 cells with pertussis toxin abolished MAP kinase activation elicited by m2 but not m1 stimulation (5), thus confirming that G proteins of the G_i family couple m2 receptors to the MAP kinase pathway. We next asked whether constitutively activated mutants of $G\alpha_q$ and $G\alpha_{12}$ were able to mimic the effect of activating the m1 and the m2 class of receptors, respectively. To that end, HA-ERK2 was co-transfected together with expression vectors carrying cDNAs for wild-type or GTPase deficient mutants of α subunits from G_{12} , G_q , and G_{13} . All transfected G protein α subunits were efficiently expressed and, as expected, expression of the activated forms of α_{12} and α_q inhibited adenylyl cyclase and potently activated phosphatidylinositol hydrolysis, respectively (data not shown). However, transfected cells expressing wild type or activated G protein- α subunits did not exhibit elevated HA-ERK2 phosphorylating activity (5). In contrast, a nearly 10 fold increase in MAP kinase activity was

observed in *v-ras* transfected cells under identical experimental conditions. Thus, expression of activated α subunits of G_q and G_{12} in Cos-7 cells failed to mimic m1 and m2-induced activation of MAP kinase, even though they elicit a subset of biochemical responses that are characteristic of each class of muscarinic receptor. We concluded that molecules in addition to activated G protein α subunits might be involved in signaling MAP kinase activation.

Agonist activated G protein-coupled receptors act as exchange factors for heterotrimeric G proteins by catalyzing the replacement of GDP by GTP bound to the α subunit, thereby causing the dissociation of α -GTP from $\beta\gamma$ subunits (6). Recently available evidence has supported an active role for the $G_{\beta\gamma}$ dimers in signal transduction (7). Thus, since activated $G\alpha$ subunits failed to mimic receptor stimulation of MAP kinase activity, we asked whether $\beta\gamma$ complexes can signal to MAP kinase by co-transfecting expression plasmids carrying cDNAs for β_1 or γ_2 subunits together with HA-ERK2. We also used an altered γ subunit lacking its isoprenylation signal. This molecule, designated γ^* , can efficiently associate with β subunits but the resulting $\beta\gamma^*$ dimers fail to translocate to the plasma membrane and are inactive in several systems (8). Whereas expression of either β or γ subunit alone had little effect on MAP kinase, coexpression of $G_{\beta\gamma}$ effectively elevated its phosphorylating activity (5). The mutated γ subunit, γ^* , neither had an effect alone nor increased MAP kinase activity when co-transfected with the β subunit, suggesting that isoprenylation of $\beta\gamma$ is required for MAP kinase stimulation. Collectively, these findings demonstrate that membrane bound $\beta\gamma$ -heterodimers directly elicit biochemical pathways leading to MAP kinase activation. Furthermore, sequestration of free $\beta\gamma$ -dimers by overexpression of G_i or by a chimeric molecule containing the extracellular and transmembrane domain of CD8 fused to the carboxyl terminal domain of β ARK, which includes the $\beta\gamma$ -binding region, abolished m2 and $\beta\gamma$ -induced activation of MAP kinase, and reduced this response when elicited by m1 stimulation (5; and unpublished observations). Thus, whereas m1 signaling to MAP kinase involves both a $\beta\gamma$ -dependent and a $\beta\gamma$ -independent pathway, m2 activation of MAP kinase appears to be strictly dependent on $G_{\beta\gamma}$.

To investigate whether signaling from m1 and m2 receptors or $\beta\gamma$ dimers involves $p21^{Ras}$ we transfected cells with plasmids expressing a dominant negative mutant of $p21^{Ras}$, N17-Ras. We found that expression of this Ras mutant nearly abolished elevation of MAP kinase activity in response to EGF receptor triggering, m1 or m2 activation, or by $\beta\gamma$ dimer overexpression (5). As another test of Ras involvement, additional Ras inhibiting constructs were tested by transfection. These included Ras-GAP, the catalytic domain of Ras-GAP and Rap-1a. Each of these constructs inhibited activation of MAP kinase by EGF and carbachol (5). In addition, we investigated the role of protein kinase C (PKC) in signaling MAP kinase activation by $G_{\beta\gamma}$ and m1 and m2 receptors, using as an approach the depletion of PKC by prolonged treatment with phorbol esters. Under these experimental conditions, the MAP kinase response to a subsequent treatment with phorbol ester was abolished, and the response elicited by m1 stimulation partially diminished (5). However, the MAP kinase stimulation by m2 stimulation or by coexpression of $\beta\gamma$ dimers was not affected by PKC depletion. Taken together, these findings strongly suggest that signaling from m1 receptors to MAP kinase involves a PKC-dependent and a PKC-independent pathway, the latter mediated by $G_{\beta\gamma}$, and that both pathways converge at the level of Ras. In contrast, signaling from m2 to MAP kinase is PKC-independent, and involves G_i - $\beta\gamma$ dimers, acting on a Ras-dependent biochemical route.

Phosphoinositide 3-kinase γ and Src-like kinases link m2 receptors to the MAP Kinase signaling pathway

Our recent findings established that m2 receptors stimulate MAPK through $G_{\beta\gamma}$ subunits, but the subsequent intervening molecules are yet to be defined. In this regard, recently available data suggest that signaling from G protein-coupled receptors to MAP kinase involves a wortmannin sensitive phosphatidylinositol-3-kinase (PI3K), and, probably, a Src-like tyrosine kinase (9,10). Several species of PI3K have been cloned and characterized. Whereas heterodimeric PI3K α and PI3K β consisting of p110 catalytic subunits and different p85 adapter molecules are regulated by tyrosine kinase receptors, a novel PI3K isotype, termed PI3K γ , can be activated by $\beta\gamma$ subunits of heterotrimeric G proteins (11). In a collaborative study with R. Wetzker's laboratory, we have recently found that expression of PI3K γ in Cos-7 cells is sufficient to stimulate MAP kinase in a wortmannin sensitive manner (12). In contrast, expression of PI3K α did not affect MAPK activity, although it elevated endogenous PIP₃ levels to an extent even great than that induced by PI3K γ (12). In addition, we have found that expression of a mutated PI3K γ that lacks lipid kinase activity abolished MAP kinase activation induced through m2 receptors or by $G_{\beta\gamma}$. Collectively, these and additional data strongly suggest that PI3K γ plays a critical role linking m2 receptors and $G_{\beta\gamma}$ to the MAP kinase signaling pathway.

Activation of m2 receptors or $G_{\beta\gamma}$ overexpression induced the tyrosine phosphorylation of Shc and its association to the adapter protein Grb2 (12), and these effects were found to be mimicked by PI3K γ expression, and sensitive to the inhibitory effect of wortmannin. Furthermore, expression of molecules acting as dominant negative mutants for Shc, Sos, Ras and Raf prevented MAP kinase activation by m2, $G_{\beta\gamma}$, or PI3K γ (12). Thus, stimulation of MAPK by PI3K γ apparently requires a tyrosine kinase which, in turn, phosphorylates Shc.

Based upon these findings we can postulate that agonist-activated m2 receptors would first cause the exchange of GDP bound to $G_{\alpha i}$ for GTP, thereby causing the dissociation of $G_{\beta\gamma}$ from GTP-bound $G_{\alpha i}$. Free $G_{\beta\gamma}$ would then recruit PI3K γ to the plasma membrane, enhancing the activity of Src or a Src-like kinase, which, in turn, would lead to the activation of the Shc-Grb2-Sos-Ras-Raf pathway, resulting in elevated MAP kinase activity (see below). The identity of the tyrosine kinases activated by m2 receptors in these cells, as well as the mechanism(s) controlling their activation warrant further investigation.

G protein-coupled receptors activate the novel Jun kinase (JNK) pathway

Accumulating evidence suggests that the G protein-coupled receptor signaling pathway converges at the level of *ras* with that emerging from receptors of the tyrosine kinase class. Thus, activation of either type of receptor would be expected to elicit a similar response at the level of nuclear transcription factors. However, we have recently observed that the activation of m1 receptors expressed in NIH 3T3 cells induced a distinct pattern of expression of immediate early genes of the *jun* and *fos* family. In particular, m1 stimulation provoked a remarkable increase in the expression of *c-jun* mRNA, and, interestingly, this response did not correlate with the activation of MAP kinases (13). Recently, it has been shown that the activity of the transcription factor *c-jun* is controlled by a novel family of enzymes structurally related but clearly distinct from MAP kinases. These enzymes, named Jun kinases (JNKs) (14), selectively phosphorylate the N-terminal transactivating domain of the c-Jun protein thereby increasing its transcriptional activity. In light of our results, we compared the ability of carbachol, PDGF and TPA to induce MAP kinase and JNK activity. In these cells carbachol, PDGF and TPA potently induced MAP

kinase activation, but only carbachol induced JNK activation (13). Thus, in NIH 3T3 cells G protein-coupled receptors but not receptors of the tyrosine kinase class can activate the JNK signaling pathway.

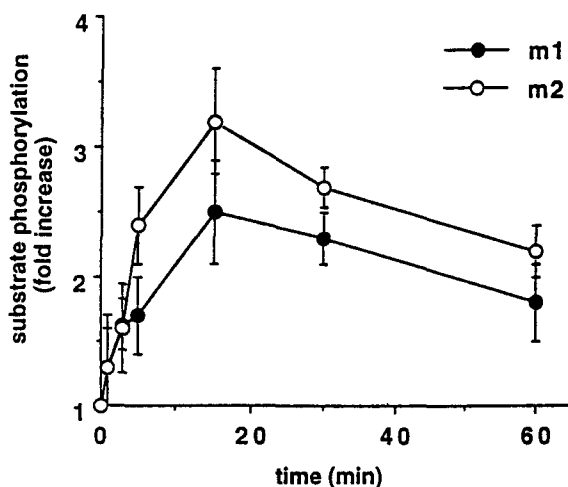


Fig. 2

Carbachol induces JNK activity in Cos-7 cells expressing m1 and m2 receptors. Cos-7 cells were transfected with expression plasmids for m1 or m2 receptors together with a plasmid expressing an epitope-tagged JNK. Cells were stimulated by addition of carbachol (100 μ M) for the indicated time, cells were lysed, and JNK activity was determined in the HA-immunoprecipitates as described (17). Data are expressed as fold increase in JNK activity with respect to non-stimulated cells. Data are from (17).

Activation of JNK involves a signaling route different from that of the Ras-MAP kinase pathway: A role for the small GTP-binding proteins Rac1 and Cdc42

Although it was initially suggested that JNK was located downstream from *ras*, this hypothesis was in conflict with the lack of activation of JNK by PDGF or by other agonists acting on receptors that are known to couple to the *ras* pathway (13). Thus, these observations suggested the existence of distinct pathways leading to the activation of either MAP kinase or JNK. To explore the mechanism of activation of JNK we expressed an epitope-tagged JNK1 (HA-JNK) in Cos-7 cells, together with activated forms of Ras, Raf and MEK. MAPK was potently activated by these mutant forms of Ras, Raf and MEK (15). However, only Ras activated JNK, although to a very limited extent (15). These observations raised the possibility that proteins other than Ras may directly regulate biochemical pathways leading to the activation of JNK. In this regard, it has been recently reported that GTP-bound forms of the Rho-related proteins Rac1 and Cdc42 can specifically associate and activate a novel serine-threonine kinase, PAK (16), a situation highly analogous to that of the Ras-Raf interaction. This observation and the failure of Ras to fully activate JNK prompted us to ask whether the Rho family of GTPases participates in signaling to the JNK pathway. In a series of experiments, we demonstrated that activated forms of Rac1 and Cdc42 can potently stimulate JNK but not MAPK activity. Thus, we proposed that whereas Ras controls the activity of MAP kinase, Rac and Cdc42 initiate a novel biochemical

route leading to JNK activation (15).

Signaling from m2 receptors to JNK involves $G_{\beta\gamma}$ subunits acting on a Ras and Rac1 dependent pathway

The mechanism of JNK activation by m1 and m2 receptors was investigated in Cos-7 cells. As shown in Figure 2, addition of carbachol to cells expressing either muscarinic receptor induced an increase in JNK activity (17). Following an experimental approach similar to that used for the study of MAP kinase activation, we showed that expression of GTPase deficient mutants of α subunits from G_q and G_{i2} failed to enhance the enzymatic activity of a cotransfected HA-JNK. In contrast, overexpression of $\beta\gamma$ subunits potently activated this enzyme (17). Furthermore, certain $\beta\gamma$ subunit-sequestering molecules prevented stimulation of JNK by either muscarinic receptor. Taken together, these findings provided strong evidence that free $\beta\gamma$ dimers rather than activated α subunits transfer signals from G protein-linked receptors to JNK.

Because we have shown that the Rho-related small GTP-binding proteins Rac1 and Cdc42 can activate JNK, we next set out to investigate whether these small GTPases mediate JNK activation by muscarinic receptors, using as an approach the expression of dominant negative mutants for Ras, RhoA, Rac1 and Cdc42. We found that coexpression of the dominant negative mutants for Rac1 and Ras prevent JNK activation by both mAChRs or when induced by $\beta\gamma$ complexes. Similarly, we have shown that JNK activation by EGF in Cos-7 cells is also blocked by N17Ras and N17Rac1 (See above). Thus, these findings support a role for Ras and Rac1 in linking both tyrosine kinase and G protein-coupled receptors to the JNK pathway.

Conclusion

The emerging picture from our studies and from recent reports is that mammalian cells have independent signaling pathways connecting cell surface receptors with each member of the MAP kinase superfamily (Fig. 3). Although Ras controls the activity of MAP kinases, Rac1 and/or Cdc42 regulate JNK activation (15). In addition, we have presented evidence supporting a role for $\beta\gamma$ subunits of heterotrimeric G proteins in communicating m1 and m2 receptors with the MAP kinase and the JNK pathway acting on, respectively, a Ras and Rac1-dependent biochemical route. Thus, taking these findings together we can postulate that $\beta\gamma$ heterodimers provide a link between heterotrimeric G proteins and small GTP-binding proteins. The molecular basis for this interaction is still poorly defined. However, it is strikingly similar to that of the pathway linking the G protein-coupled pheromone receptors to MAP kinase related enzymes in the budding yeast *S. cerevisiae*. In yeast, the G protein β subunit can initiate activity of a MAP kinase cascade by binding an exchange factor for the Cdc42 GTPase, and then this GTP-binding protein physically interacts with the most upstream kinase, Ste20, causing its activation (18). Thus, our recent observations that m1 and m2 receptors communicate to MAP kinase and JNK through $\beta\gamma$ complexes in a Ras and/or Rac1 dependent manner might represent a biologically relevant example of a signal transduction pathway extraordinarily conserved from yeast to mammals. This also provides an attractive model to elucidate the nature of those molecules linking heterotrimeric G proteins to at least two independent kinase cascades connecting the cell surface to nucleus, thus controlling the expression of genes involved in many cellular processes, including cell proliferation, differentiation, programmed cell death, as well as in a number of differentiated functions.

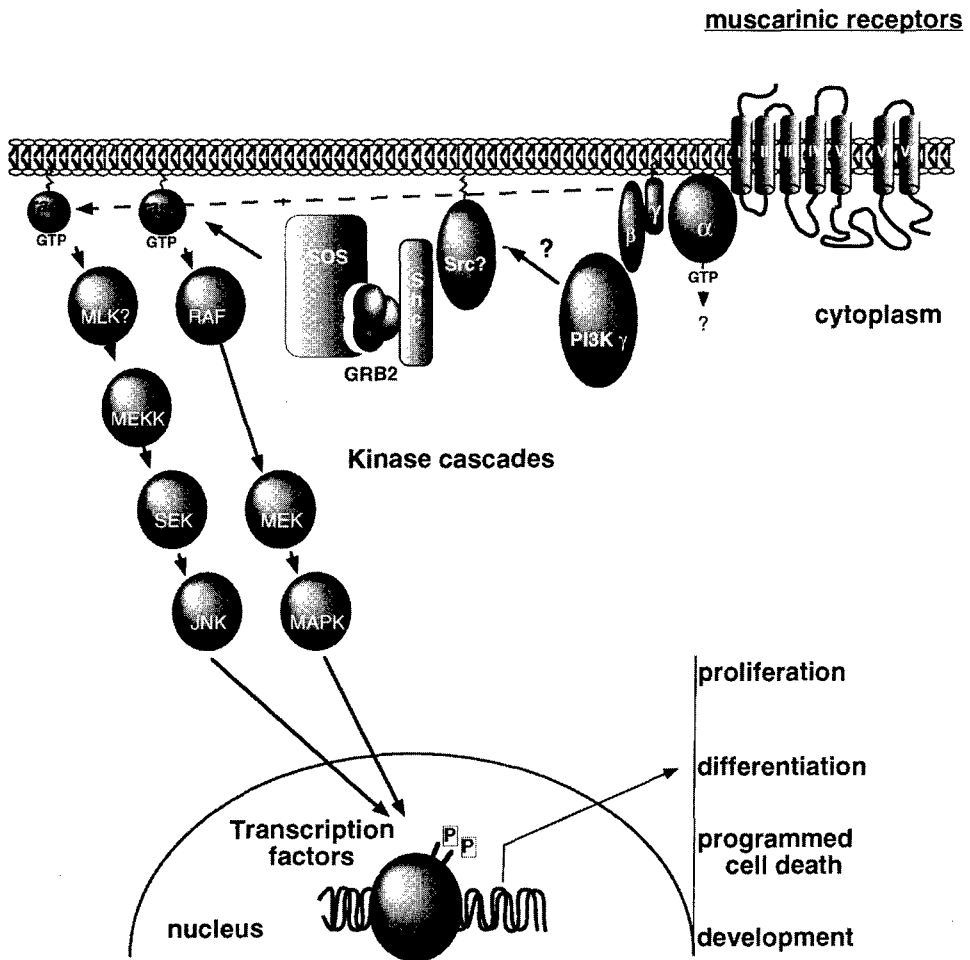


Fig. 3

Signaling through muscarinic receptors to MAP kinase and JNK in Cos-7 cells involves $\beta\gamma$ subunits acting on Ras and Rac1. Available data suggest that the pathway connecting $G_{\beta\gamma}$ to Ras involves the γ isotype of PI3K, a tyrosine kinase of the Src family, Shc, Grb2 and Sos. Molecules linking $G_{\beta\gamma}$ to Rac1 are yet to be identified. Ras controls MAP kinase by acting directly on Raf, leading to the activation of MEK and MAP kinase. The small GTP-binding proteins Rac1 and Cdc42 regulate the JNK pathway, probably through the activation of a member of the MLK family of kinases (19) and, sequentially, MEKK and SEK. See text for details.

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STRUCTURAL BASIS OF RECEPTOR/G PROTEIN COUPLING SELECTIVITY STUDIED WITH MUSCARINIC RECEPTORS AS MODEL SYSTEMS

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Summary

Different muscarinic acetylcholine receptor subtypes were used as model systems to study the structural basis of receptor/G protein coupling selectivity. Extensive mutagenesis studies have previously led to the identification of single amino acids on the m3 muscarinic receptor protein (located in the second intracellular loop (i2) and at the N- and C-terminus of the third intracellular loop (i3)) that dictate selective recognition of Gq/11 proteins by this receptor subtype. Based on these results, we proposed a model of the intracellular m3 receptor surface in which the functionally critical residues project into the interior of the transmembrane receptor core. To identify specific regions on the G protein(s) that are contacted by these different, functionally critical receptor sites, we recently employed a novel experimental strategy involving the coexpression of hybrid m2/m3 muscarinic receptors with hybrid G α -subunits. Using this approach, we could demonstrate that the C-terminus of G protein α_i/o -subunits is recognized by a short sequence element in the m2 muscarinic receptor ("VTIL") that is located at the junction between the sixth transmembrane domain (TM VI) and the i3 loop. We could show that this interaction is critically involved in determining coupling selectivity and triggering G protein activation. By using a similar strategy (coexpression of mutant muscarinic receptors with hybrid G α -subunits), other major receptor/G protein contact sites are currently being identified. These studies, complemented by biochemical and biophysical approaches, should eventually lead to a detailed structural model of the ligand-receptor-G protein complex.

Key Words: site-directed mutagenesis, G protein-coupled receptors, G protein α -subunits

The five muscarinic acetylcholine receptors (m1-m5) are typical members of a superfamily of plasma membrane receptors which transmit extracellular signals such as neurotransmitters or hormones into the cell interior via coupling to heterotrimeric G proteins (1,2). Following ligand binding, these receptors are predicted to undergo as yet poorly defined conformational changes that enable the receptor to interact with specific classes of G proteins, which are attached to the cytoplasmic side of the membrane. In most cases, each individual receptor can couple only to a distinct subset of the many structurally similar G proteins expressed within a cell (3-5). To understand how this selectivity is achieved at a molecular level has become the major focus of a great number of laboratories, and is also the topic of this chapter.

For many years we have used different members of the muscarinic receptor family as model systems to study the molecular mechanisms governing receptor/G protein coupling selectivity (for a recent review, see ref. 6). Based on their differential coupling properties, the five muscarinic recep-

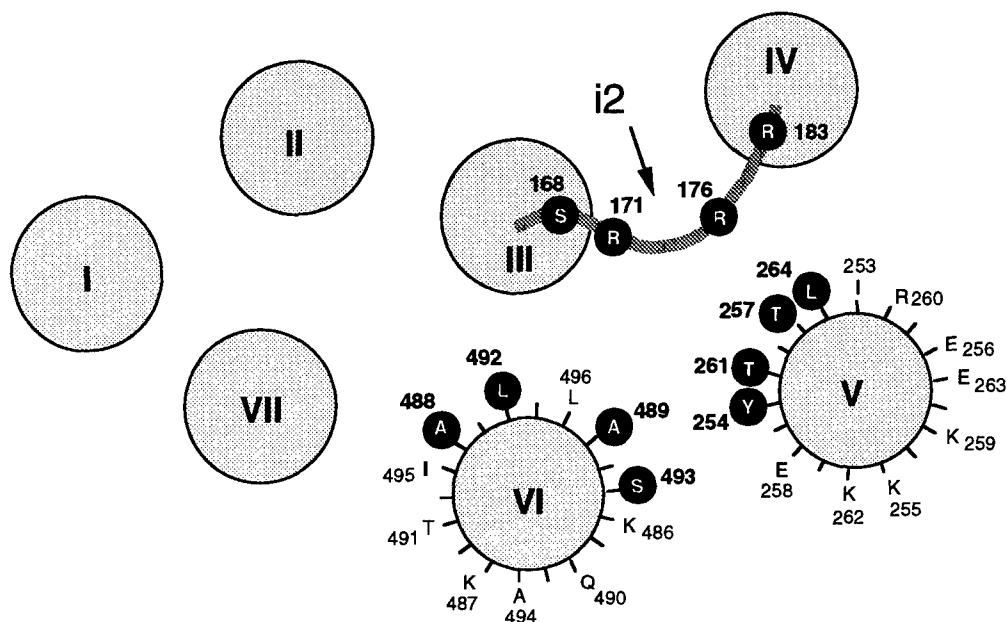


Fig. 1

Model of the intracellular surface of the m3 muscarinic receptor displaying residues critical for proper G protein recognition. The arrangement and orientation of the seven transmembrane helices (I-VII), shown here as seen from the cell interior, is based on the "Baldwin projection" (23,24). Numbers refer to amino acid positions in the rat m3 muscarinic receptor (33). The importance of the highlighted amino acids for selective coupling of the m3 receptor to Gq/11 proteins has been demonstrated in previous mutagenesis studies (15,17,18,21). According to the model shown here, the functionally important residues at the N- and C-terminus of the i3 loop are predicted to form two adjacent hydrophobic patches which, together with several hydrophilic residues in the i2 loop, can dictate the selectivity of receptor/G protein interactions.

tors can be classed into two major functional categories (7-10): the odd-numbered muscarinic receptors, m1, m3, and m5, preferentially couple to G proteins of the Gq/G11 family, resulting in the stimulation of different isoforms of phospholipase C β and the breakdown of phosphatidylinositol (PI) lipids. The m2 and m4 receptors, on the other hand, are primarily linked to G proteins of the Gi/G0 class, which, at a biochemical level, mediate the inhibition of adenylyl cyclase.

Receptor residues critical for coupling selectivity

By analyzing the functional properties of hybrid receptors constructed between functionally different muscarinic receptor subtypes, we (11,12) and others (13,14) demonstrated that the N-terminal segment of the i3 loop plays a key role in determining these coupling differences. Based on a more systematic analysis of a larger number of hybrid receptors, we subsequently showed that the i2 loop and the C-terminal segment of the i3 domain also significantly contribute to proper G protein recognition (15). We found that these regions act in a cooperative fashion to dictate the specificity of receptor/G protein coupling (15). These findings obtained with the muscarinic receptors are in good agreement with a considerable number of biochemical and mutational studies on several other G

protein-coupled receptors such as rhodopsin and the adrenergic receptors (3,4,16).

Based on these results, we were then able to identify a set of single amino acids (located in the i2 loop and the membrane-proximal portions of i3) that quantitatively determine the coupling profile of the m3 muscarinic receptor (15,17,18). We could show that substitution of these residues (highlighted in Fig. 1) into the m2 muscarinic receptor yields a mutant receptor that is functionally very similar to the wild type m3 receptor (15). Consistent with this finding, these residues are highly conserved in the m1 and m5 muscarinic receptors, which, like the m3 receptor, are also selectively linked to G proteins of the Gq/11 family. Considerable evidence (19-22) suggests that the N- and C-terminal segments of the i3 loop form α -helical extensions of TM V and VI, respectively. Based on this concept, a model of the intracellular receptor surface of the m3 receptor is shown in Fig. 1.

The arrangement and orientation of the seven transmembrane helices shown in Fig. 1 (as seen from the cell interior) is essentially based on the model proposed by J. Baldwin (23,24). The Baldwin projection is based on low resolution electron diffraction data of rhodopsin (25) and several other theoretical considerations which cannot be detailed here. In this model (Fig. 1), the functionally important residues at the N- and C-terminus of the i3 loop are predicted to form two adjacent hydrophobic patches that project into the interior of the receptor protein. Our data with the muscarinic receptors suggest that these two hydrophobic surfaces, together with several hydrophilic residues in the i2 loop, are sufficient to determine proper G protein recognition.

Identification of a specific receptor/G protein contact site

Based on these results, our next goal was to identify the specific regions on the G protein(s) that are in contact with these distinct, functionally important receptor sites. Such information is clearly essential for understanding the molecular basis of receptor/G protein coupling selectivity and G protein activation. Biochemical and molecular genetic studies (26,27) have identified several regions on the G protein α -subunits that are predicted to directly contact the receptor protein (note, however, that these putative receptor recognition regions are distinct from those G α domains involved in the activation of effector enzymes, such as adenylyl cyclase or phospholipase C; refs. 26,27). Specifically, several lines of evidence suggest that the C-terminus of G α , a region that has been implicated most consistently in receptor/G protein coupling, plays a key role in triggering receptor-mediated G protein activation (26-28).

To identify the receptor site that can interact with the C-terminus of G α , we took advantage of the following observation: consistent with its known G protein coupling preference, the m2 muscarinic receptor, a prototypical Gi/o-coupled receptor, does not interact with wild type Gq to a significant extent, and therefore does not stimulate PI hydrolysis efficiently (Fig. 2). However, as has been observed with other Gi/o-coupled receptors (29), the m2 receptor can efficiently interact with mutant G α q subunits in which the last five amino acids of G α q are replaced with the corresponding G α i or G α o sequences, resulting in a pronounced stimulation of the PI cascade (ref. 30; Fig. 2). These data suggest that the m2 receptor contains a sequence element that can be specifically recognized by the C-terminus of G α i/o-subunits.

By analyzing a large number of mutant muscarinic receptors, we could show (30) that the ability of the m2 receptor to interact with such hybrid G proteins specifically depends on the presence of a four-amino-acid motif (Val385, Thr386, Ile389, and Leu390; hereafter referred to as VTIL motif) located at the i3 loop/TM VI junction (Fig. 3). We showed, for example, that replacement of these four m2 receptor residues with the corresponding residues present in the Gq/11-coupled muscarinic receptors virtually abolished the ability of the resulting mutant receptor (m2(VTIL→AALS)) to functionally interact with wild type G α i2 (30) or with mutant G α q subunits containing α i or α o

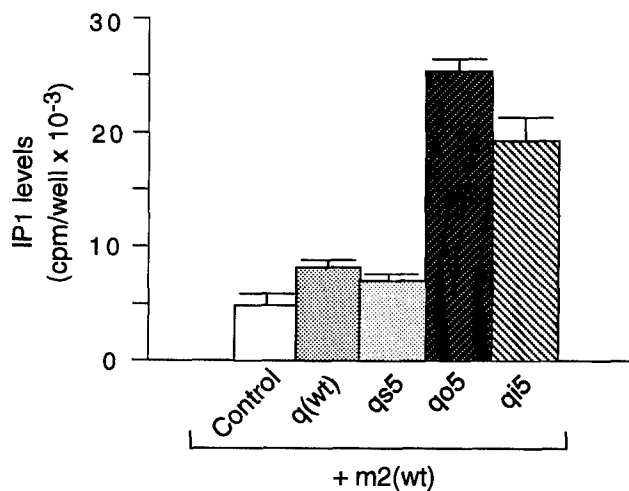


Fig. 2

Functional interaction of the wild type m2 muscarinic receptor (m2(wt)) with wild type and mutant G protein α_q -subunits. COS-7 cells were cotransfected with expression plasmids coding for m2(wt) and wild type $G\alpha_q$ (q(wt)) or mutant $G\alpha_q$ -subunits in which the C-terminal five amino acids of q(wt) (EYNLV) were replaced with the corresponding sequences from $G\alpha_{i2}$ (qi5; *DCGLF*), $G\alpha_o$ (qo5; *GCGLY*), or $G\alpha_s$ (qs5; *QYELL*). Control IP₁ levels were determined in cells coexpressing m2(wt) and q(wt) in the absence of carbachol. Cells were stimulated with the agonist carbachol (1 mM), and increases in intracellular IP₁ levels were determined (data taken from ref. 30).

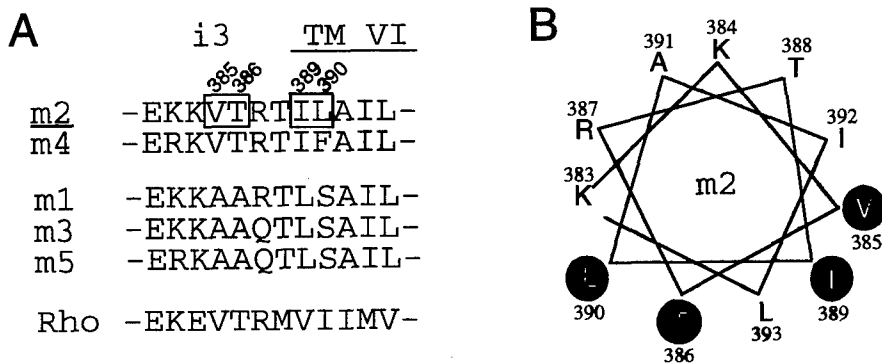


Fig. 3

(A) Comparison of m1-m5 muscarinic receptor sequences at the i3 loop/TM VI junction. The four m2 receptor residues (Val385, Thr386, Ile389, and Leu390) known to be critically involved in G protein recognition are boxed (30,31). Numbers refer to amino acid positions in the human m2 muscarinic receptor (33). For comparison, the corresponding rhodopsin (Rho) sequence is also shown. The single-letter amino acid code is used. (B) Helical wheel representation of the m2 receptor sequence at the i3 loop/TM VI junction, as viewed from the cytoplasm. The residues predicted to form the functionally important VTIL motif (30,31) are highlighted.

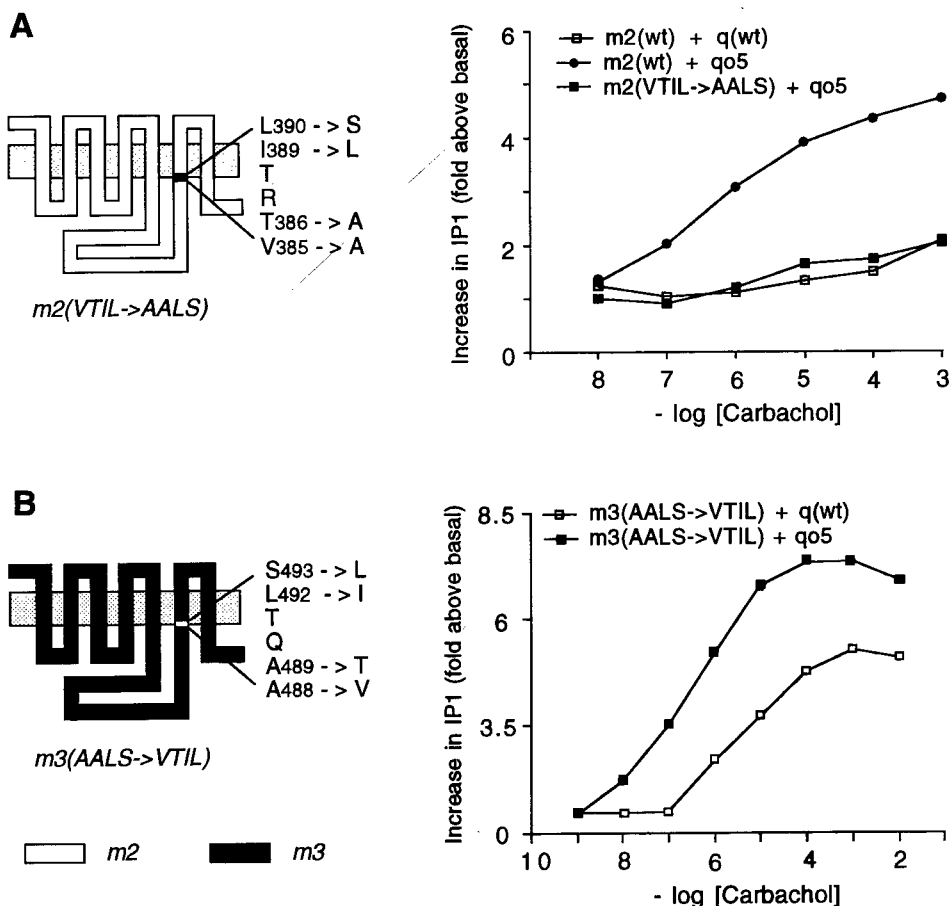


Fig. 4

Functional interaction of the *m2(VTIL->AALS)* (A) and *m3(AALS->VTIL)* (B) mutant muscarinic receptors with wild type $G\alpha_q$ (*q(wt)*) and the mutant $G\alpha_q$ -subunit, *qo5*. In *m2(VTIL->AALS)* and *m3(AALS->VTIL)*, Val385, Thr386, Ile389, and Leu390 (*m2* receptor sequence) and Ala488, Ala489, Leu492, and Ser493 (*m3* receptor sequence) were exchanged between the two wild type receptors. In *qo5*, the C-terminal five amino acids of *q(wt)* (*EYNLV*) were replaced with the corresponding $G\alpha_o$ sequence (*GCGLY*). COS-7 cells coexpressing receptor and G protein constructs were incubated with the indicated concentrations of carbachol, and increases in intracellular IP1 levels were determined as described (30). A representative experiment is shown; two additional experiments gave similar results.

sequence at their C-terminus (Fig. 4A). On the other hand, substitution of the VTIL motif into the wild type *m3* receptor resulted in a hybrid receptor (*m3(AALS->VTIL)*) that, when coexpressed with mutant $G\alpha_q$ -subunits containing α_i or α_o sequence at their C-terminus, was able to stimulate PI hydrolysis with increased efficacy and efficiency (as compared with wild type $G\alpha_q$; Fig. 4B). Moreover, this mutant receptor gained the ability to mediate inhibition of adenylyl cyclase when coexpressed with wild type $G\alpha_{i2}$, in a fashion similar to the wild type *m2* receptor (30).

The most straightforward explanation for these findings is that the VTIL motif can functionally interact with the C-terminus of $G\alpha_i/o$ -subunits and that this interaction is intimately involved in determining coupling selectivity and triggering G protein activation. This motif is reasonably well conserved in many other G_i/o -coupled receptors such as the α_2 -adrenergic, somatostatin, or opioid receptors (30). It should be noted that the corresponding region at the i3 loop/TM VI junction in the photoreceptor, rhodopsin, shares a high degree of sequence identity with the corresponding m2 (m4) muscarinic receptor sequence (Fig. 3A). For example, Val250 and Thr251 in (bovine) rhodopsin directly correspond to Val385 and Thr386 in the m2 receptor (Fig. 3A). Interestingly, a site-directed spin labeling study (in which individual residues at the i3 loop/TM VI junction in rhodopsin were replaced with cysteine residues, followed by covalent modification of these cysteine residues with a spin marker) recently provided direct structural evidence that this region (that includes Val250 and Thr251) does in fact adopt an α -helical secondary structure (22). Given the high degree of sequence identity between rhodopsin and the m2 (m4) muscarinic receptor within this region, it is highly likely that Val385, Thr386, Ile389, and Leu390 form a contiguous (hydrophobic) surface located on one side of an amphiphilic α -helix (Fig. 3B).

Interestingly, we recently showed that the insertion of one or more extra alanine residues immediately C-terminal of the VTIL motif into the m2 muscarinic receptor led to mutant receptors that could activate the proper G proteins even in the absence of agonists (31). We could further demonstrate that the constitutive activity displayed by these mutant receptors was strictly dependent on the structural integrity of the VTIL motif. On the other hand, deletion of the alanine residue C-terminal of the VTIL motif (Ala391; Fig. 3A) in the wild type m2 receptor completely abolished receptor activity (in the absence or presence of agonist ligands; ref. 31). These data are consistent with a model of receptor activation that involves a ligand-induced rotational and/or translational movement of TM VI, thus exposing the functionally critical residues at the i3 loop/TM VI junction for interactions with the C-terminus of $G\alpha_i/o$ -subunits.

According to the Baldwin projection (23,24), Val385, Thr386, and Ile389 are predicted to project into the interior of the transmembrane receptor core where they may be engaged in tertiary interactions with residues located on other transmembrane helices. Interestingly, spin labeling studies have shown that several residues at the i3 loop/TM VI junction in bovine rhodopsin (particularly Val250 and Thr251) undergo large increases in mobility upon photoexcitation (22). In addition, studies with mutant rhodopsin molecules modified with two different spin markers (32) recently showed that this mobility increase is most likely due to a ligand-induced rotational movement of TM VI (accompanied by an "outward" movement of this TM helix). These findings, therefore, provide direct experimental support for our previously proposed model of ligand-induced receptor activation (refs. 30, 31; see also previous paragraph).

Detailed structural analysis of a receptor/G protein contact site

To further investigate the structural requirements for the functional interaction of the VTIL motif in the m2 muscarinic receptor with the C-terminus of $G\alpha_i/o$ -subunits, Val385, Thr386, Ile389, and Leu390 were systematically replaced, either individually or in combination, with the corresponding residues present in the $G_q/11$ -coupled muscarinic receptors. The ability of the resulting mutant m2 receptors to interact with a mutant $G\alpha_q$ -subunit in which the last five amino acids were replaced with α_o sequence (qo5) was investigated in cotransfected COS-7 cells. Preliminary data suggest that the presence of three of the four targeted m2 receptor residues (Val385, Thr386, and Ile389) is absolutely essential for efficient recognition of C-terminal $G\alpha_i/o$ sequences. On the other hand, Leu390 does not seem to play a critical role in determining the specificity of this interaction (E. Kostenis and J. Wess, unpublished results). It is likely that Val385, Thr386, and Ile389 are engaged in specific interactions with the C-terminus of $G\alpha_i/o$ -subunits, and that each of these contact sites contributes to the specificity and efficiency of receptor/G protein coupling.

To study which specific amino acids within the C-terminal segment of G α_i/o -subunits are critical for this interaction to occur, the wild type m2 receptor was coexpressed with a series of mutant G α_q -subunits containing single or multiple $\alpha_q \rightarrow \alpha_i1,2$ point mutations at their C-terminus. Remarkably, the wild type m2 receptor, while unable to efficiently stimulate wild type G α_q , gained the ability to productively interact with three G α_q single point mutants (E. Kostenis, B. Conklin, and J. Wess, unpublished results). In these (mouse) mutant G α_q constructs, Tyr350 (position -4), Asn351 (position -3), or Val353 (position -1) were replaced with Cys, Gly, or Phe, respectively. The ability of these mutant G α_q -subunits to productively interact with the wild type m2 receptor may be indicative of the fact that each of the three introduced $\alpha_i1,2$ residues can participate in specific interactions with the functionally critical amino acids at the i3 loop/TM VI junction in the receptor protein. These studies provide the first example that the receptor coupling selectivity of G protein α -subunits can be switched by single amino acid substitutions.

Future outlook

Encouraged by these results, we are currently employing a similar strategy (coexpression of wild type and mutant muscarinic receptors with hybrid G α -subunits) to identify other major receptor/G α contact sites. Preliminary data suggest that a short segment at the N-terminus of G α_i may also play an important role in dictating proper receptor/G protein recognition (E. Kostenis, Christian Lerche, B. Conklin and J. Wess, unpublished results). To determine the receptor site that can recognize this region of G α_i , the ability of a series of mutant m2 muscarinic receptors to interact with mutant G α_q -subunits containing G α_i sequence at their N-termini is currently being explored. These studies, which can also be extended to other classes of G protein-coupled receptors or G protein α -subunits, should eventually lead to the delineation of three-dimensional models of the receptor/G protein interface, thus providing valuable clues regarding the molecular mechanisms involved in receptor-mediated G protein activation.

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REGULATION OF MUSCARINIC M₂ RECEPTORS

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Summary

The molecular mechanisms involved in the regulation of muscarinic receptor gene expression are poorly understood. In an effort to gain a better understanding of the regulation of M₂ receptors, we have investigated homologous and heterologous regulation of M₂ muscarinic receptor protein and gene expression in human embryonic lung fibroblasts (HEL 299 cells). HEL 299 cells constitutively express m2 receptors, with no evidence of other muscarinic receptor subtypes. We have shown that M₂ receptors in these cells can be down-regulated by muscarinic and β_2 -adrenergic receptor agonists. Unlike the down-regulation mediated by muscarinic and β -adrenergic stimulation, activation of PKC with PDBu was mediated through changes in m2 muscarinic receptor mRNA through reduced gene transcription. Because of the inflammatory nature of asthma, we have focused on delineating the interactions between cytokines and M₂ receptors in an attempt to define potential endogenous modulators of M₂ receptor expression. We have shown that the multi-functional cytokine, transforming growth factor β_1 (TGF- β_1), which is involved in several inflammatory conditions induces desensitization and down-regulation of M₂ muscarinic receptor protein and gene expression that was mediated through a reduction in the rate of m2 receptor gene transcription. Other cytokines of interest are tumor necrosis factor α (TNF- α) and interleukin 1 β (IL-1 β) which are elevated in asthma. We have demonstrated that TNF- α and IL-1 β synergise to induce down-regulation of M₂ muscarinic receptor protein and mRNA which was associated with functional desensitization of the receptor protein. The M₂ receptor mRNA down-regulation appeared to be mediated through a reduction in the rate of m2 receptor gene transcription which may be dependent on the transcription and translation of unknown protein factor(s). Moreover, a role of PKA and ceramide pathways in M₂ receptor regulation is suggested. Collectively, our work provides a mechanistic explanation of previous reports indicating altered function of M₂ receptors in asthma. Our results also suggest that the expression of this receptor subtype may be under the control of a cytokine network at the airways.

Key Words: M₂ receptors, cytokines, protein kinase C, MAP kinase

Of the five cloned muscarinic receptor subtypes, human lung expresses only three receptor subtypes (M₁, M₂ and M₃) that are differentially distributed in the airways (1-3). M₁-receptors are localized to alveolar walls, parasympathetic ganglia and submucosal glands, whereas M₃ - receptors are predominant in airway smooth muscle and submucosal glands. By contrast M₂-receptors are localized to airway smooth muscle of small airways, although there is little evidence that these receptors play an important functional role on the regulation of airway smooth muscle tone, at least in human airways (4). Functional studies suggest that M₂-receptors play an important role in regulating the release of acetylcholine from parasympathetic

nerves in the airways (5). M₂-receptors which inhibit cholinergic neural contraction have been demonstrated in functional studies of human airways *in vitro* and recent studies have confirmed that pre-junctional M₂-receptors inhibit the release of acetylcholine in human airways (6). There is some evidence that M₂-receptors may be dysfunctional in patients with asthma (7-9) and this might contribute to exaggerated cholinergic reflex bronchoconstriction in asthma. There are several possible mechanisms that might result in dysfunction of M₂-receptors, including viral infections, eosinophil basic proteins and oxidants (10). The inflammatory process may also result in impaired function of M₂-receptors

Little is known about the factors that regulate M₂-receptors, however. Because it is not possible to examine molecular mechanisms in parasympathetic nerves, we have chosen a human cell line that expresses M₂-receptors in the absence of M₁- and M₃-receptors, namely human embryonic lung fibroblasts (HEL 299 cells). We have studied the effects of cholinergic agonists, cyclic AMP and various inflammatory cytokines on the binding characteristics, coupling and gene expression of M₂-receptors in this cell line.

Effect of cholinergic agonists

Specific [³H]NMS and [³H]QNB binding to muscarinic receptors in HEL 299 cells was best described by the interaction of the radioligands with a single population of high affinity binding sites. The cholinergic agonist carbachol (1mM) induced a time-dependent decrease in the number of muscarinic receptors measured with the hydrophilic ([³H]NMS) and the lipophilic ([³H]QNB) ligands without any change in the affinity of the remaining binding sites (11). This suggests that the detected receptor down-regulation is due to a decrease in receptor density and not a result of the presence of residual agonist in the binding assay. The loss of the lipophilic [³H]QNB binding sites during the first 2 h of carbachol treatment occurred at slower rate than does the loss of [³H]NMS binding sites as a result of receptor sequestration. Within 12 h, the process had approached steady state with 40 to 60% loss of receptors. The down-regulation seen after long-term carbachol treatment is probably due to receptor degradation triggered by prolonged carbachol occupancy. This down-regulation was accompanied by uncoupling of the M₂-receptors after 24 h carbachol treatment. Our results also suggest that homologous sequestration, desensitization and down-regulation of muscarinic M₂-receptors does not involve transcriptional or post-transcriptional modification of m2 receptor mRNAs. There was no reduction in either m2-receptor mRNA stability, nor in m2-receptor gene transcription measured by nuclear run-on assay (11). This is in contrast to a reduction in m2 receptor mRNA reported after carbachol incubation in cultured neuronal cells (12).

Cross-talk between muscarinic M₂ and β_2 -adrenergic receptors

We have shown that HEL 299 cells express functional β_2 -adrenergic receptors in HEL 299 cells. The β -adrenergic antagonist [¹²⁵I]iodopindolol recognized a single population of binding sites on these cells with a K_D value and binding capacity of 20.9 ± 1.7 pM and 49.6 ± 6.6 fmol/mg protein respectively. These receptors display characteristic properties of the β_2 -adrenoceptor subtype with regard to ligand binding, functional response (cAMP accumulation) and mRNA expression.

The presence of functional M₂ and β_2 -adrenergic receptors in HEL 299 cells enabled us to investigate any possible cross-talk between these two receptors. Carbachol (1mM) treatment had no effect on the density or the affinity of β_2 -adrenoceptors in these cells. However, short-term incubation with the long acting β_2 -adrenergic receptor agonist, procaterol (5 μ M), induced sequestration of the muscarinic receptors followed by their recycling to the cell surface before subsequent down-regulation (13). Down-regulation was accompanied by functional uncoupling of the M₂ receptors, as inhibition of cAMP accumulation by carbachol was lost after 24 h treatment with procaterol. Down-regulation in receptor number was not a consequence of

changes in m2 gene expression as 24 h procaterol treatment did alter m2 mRNA levels, although shorter treatments resulted in a modest (25%) but significant, increase in m2 mRNA between 0.5 and 2 h. The loss in receptor density appeared to be cAMP-dependent, as it was mimicked by forskolin and by the cAMP analog 8-Br-cAMP. The cellular kinases, protein kinase A (PKA) and protein kinase C (PKC) are also implicated in the down-regulation and desensitization process, as selective inhibitors of both PKA (H-8) and PKC (GF 109203X) fully and partially attenuated down-regulation and desensitization respectively (13).

Effect of protein kinase C activation

Direct stimulation of PKC with the phorbol ester phorbol 13,14-dibutyrate (PDBu: 100 nM) resulted in a time-dependent decrease in [³H]NMS binding and the steady state levels of m2-muscarinic receptor mRNA and led to a functional uncoupling of M₂ receptors (14).

The loss of m2-receptor mRNA and protein in HEL 299 cells after exposure to PDBu appears to be a PKC-mediated effect since pre-treatment with the PKC inhibitor GF completely inhibited the PDBu-induced reduction in m2-receptor mRNA and significantly inhibited the reduction in M₂-receptors. Incubation with the inactive 4 α -PDBu (100 nM), confirmed a PKC-mediated effect as 24 h treatment had no effect on [³H]NMS binding or m2-receptor mRNA levels. Potential PKC desensitization following long-term treatment with PDBu was not observed as the calcium ionophore A23187, which is thought to potentiate the effect of PKC stimulation, in combination with PDBu did not produce any further down-regulation of M₂ muscarinic receptor protein or mRNA. This result indicates a relative insensitivity of PKC present in HEL 299 to calcium which may relate to a particular PKC isoform in these cells (15). Elevation of intracellular Ca²⁺ by the ionophore A23187 had no effect on the [³H]NMS binding capacity and on the level of muscarinic m2 mRNA. This result argues against the involvement of the Ca²⁺-calmodulin-dependent protein kinase in the down-regulation of M₂-receptors.

The reduction in muscarinic m2-receptor mRNA was not due to post-transcriptional modification of the mRNA but rather was mediated through a reduction in the rate of transcription of the m2-receptor gene, as measured by nuclear run-on assays. Furthermore, this down-regulation requires protein synthesis as the translation inhibitor cyclohexamide (10 μ g/ml) protected against receptor down-regulation. Thus, synthesis of at least one other protein factor is required after PKC stimulation to alter m2-receptor mRNA levels. The nature of the protein(s) induced by PKC activation are not known. However, PKC is known to phosphorylate and induce DNA binding activity of a number of proteins, including transcription factors such as nuclear factor- κ B (NF- κ B) and activator protein 1 (AP-1), which may in turn alter the expression of other genes (16,17).

Recently, several interactions between PKC and mitogen-activated protein (MAP) kinases have been identified and in particular the Raf-1-activated pathway that results in the activation of extracellular-signal regulated kinases (ERK1, ERK-2) (18). ERK activation is selectively blocked by the inhibitor PD 098059, which inhibits MAPKK (MEK) (19). This inhibitor partially blocked the phorbol ester reduction in m2-receptor mRNA, indicating that some of the effects of PKC activation are mediated via MAP kinase and ERK activation (20).

Effect of cytokines

Transforming growth factor- β

Transforming growth factor β (TGF- β) occurs as a group of disulfide-linked proteins comprising 12.5 kDa homodimers which are synthesized and secreted by most cell types as latent high molecular weight complexes (21). They exert their action by binding to specific cell surface serine/threonine kinase receptors. TGF- β 1 has important physiological roles in the regulation of

embryogenesis, tissue repair, inflammation or cell adhesion, growth and differentiation. In an effort to gain a better understanding of the regulation of muscarinic receptor expression, particularly in inflammatory diseases, we investigated the effect of TGF- β 1 on the gene expression of M₂ muscarinic receptors in HEL 299 cells.

In human embryonic lung fibroblasts, TGF- β 1 induced a time-dependent down-regulation of M₂ muscarinic receptor binding sites as measured by [³H]NMS. This down-regulation was slow with 58% loss of total receptors after 24 h of treatment. The affinity of [³H]NMS for the remaining sites was unaltered by TGF- β 1. Northern blot analyses showed a 72% decrease in the steady-state levels of m2 muscarinic receptor mRNA following TGF- β 1 treatment for 24 h (22). The loss of [³H]NMS binding sites occurred slowly, which reflects a fall in the steady-state levels of m2-receptor mRNA, rather than internalization of the receptors through phosphorylation. The delay between protein loss and the fall in mRNA levels may be indicative of the rate of receptor turnover within the cell. The TGF- β 1 effect was long lasting ($t_{1/2} \sim 8$ h) as at least 12 h was required for m2-receptor mRNA to return to basal levels after TGF- β 1 washout. Previous results obtained in the same cell line have shown that the recovery of M₂-receptor protein after receptor alkylation was mainly through the synthetic pathway with an estimated half-life of receptor synthesis around 12 h (23). The loss in [³H]NMS binding was accompanied by a reduced adenylyl cyclase activity and functional desensitization of M₂ muscarinic receptors. There was no effect of TGF- β 1 on the muscarinic m2-receptor mRNA half-life measured in the presence of actinomycin D, but rate of m2-receptor gene transcription measured with nuclear run-on assay was reduced by 50%, indicating reduced gene transcription (22).

We have shown that there is requirement for *de novo* protein synthesis for receptor down-regulation. The nature of the protein(s) induced by TGF- β 1 activation is not known. However, TGF- β 1 is known to induce DNA binding activity of a number of proteins, including transcription factors such as AP-1. Electrophoretic DNA mobility shift assays showed a rapid and concomitant increase in AP-1 and NF- κ B but not OCT-1 DNA binding activity to nuclear extracts from cells treated with TGF- β 1 stimulation (22). This increase peaked at 15-30 min after treatment and declined to control levels thereafter. The anti-oxidant pyrrolidine dithiocarbamate significantly repressed the induction of NF- κ B but not AP-1 by TGF- β 1. The same treatment provided a significant protection against TGF- β 1-induced down regulation of M₂-receptor protein and gene expression. These results suggest the involvement, at least in part, of the transcription factor NF- κ B in the down-regulation process. However, our results do not rule out the involvement of other transcription factors. Indeed, the kinetics of AP-1 and NF- κ B induction by TGF- β 1 was very rapid, suggesting activation of DNA binding of pre-existing molecules, rather than the occurrence of *de novo* protein synthesis. On the other hand, the cycloheximide data suggests that there is a requirement for protein synthesis for receptor down-regulation. Direct interactions of these transcription factors with the m2-receptor gene promoter can not be measured directly as no sequence data is available to date.

Proinflammatory cytokines

Cytokines released by immune and inflammatory cells infiltrating the airways are well recognized as key mediators in the orchestration and perpetuation of the chronic inflammation in asthma (24). The proinflammatory cytokines, tumor necrosis factor α (TNF- α) and interleukin-1 β (IL-1 β) are increased in asthma. Stimulation of HEL 299 cells with TNF- α or IL-1 β had no effect on M₂ muscarinic receptor expression. However, the combination of these two cytokines markedly down-regulated muscarinic M₂ receptor protein and uncoupled M₂ receptors from adenylyl cyclase (25). The effect of TNF- α and IL-1 β on M₂ muscarinic receptor protein could be extended to mRNA. Whereas down-regulation of m2 muscarinic receptor mRNA was absent to either TNF- α or IL-1 β alone, there was a dramatic and sustained decrease in down-regulation of m2 mRNA when the two cytokines were administered in combination. The m2 muscarinic receptor mRNA steadily decreased over time, apparent after 4 h of stimulation and reached a plateau of 89% control at 14 h and was stable up to 24 h. There was no effect of

TNF- α and IL-1 β on the m2 muscarinic receptor mRNA stability and nuclear run-on assays showed a reduced m2 receptor gene transcription. Sequential cytokine addition suggests that the synergy involves post-receptor events.

To characterize the intracellular signaling pathways leading to receptor down-regulation, we have investigated the involvement of PKA and PKC in this process. While the PKA inhibitor H-8 provided a significant protection against receptor down-regulation, the PKC inhibitor GF109203X had no effect. Beside the classical cyclic AMP and PKC pathways, a third phosphorylation pathways known to be activated by these cytokines is represented by the lipid second messenger ceramide. IL-1 β and TNF- α rapidly increase the cellular content of ceramide produced following the hydrolysis of sphingomyelin by two types of sphingomyelinases, a membrane-associated neutral and an endosomal acidic sphingomyelinase (26). Treatment of HEL 299 cells with N-acetyl-sphingosine (or C2-ceramide), a cell-permeable analog of natural ceramide, did not affect the steady-state levels of m2 muscarinic receptor mRNA over the time-course investigated, in an analogous manner to TNF- α and IL-1 β alone. However, the combination of C2-ceramide either with TNF- α or IL-1 β markedly down-regulated m2 receptor mRNA expression after 24 h of treatment to a comparable extent to that produced by the combination of the two cytokines. These results are consistent with a role for ceramide pathway in m2 receptor down-regulation induced by the combination of TNF- α and IL-1 β treatment.

A further downstream signaling event known to be triggered by TNF- α and IL-1 β is activation of the MAP kinase cascade which comprises the extracellular signal-regulated kinases (ERK) and the c-Jun N-terminal protein kinases (JNK) (18). Using an "in gel kinase assay" we have shown that TNF- α and/or IL-1 β activated the JNK-46 and JNK-55 and to a lesser extent p42 and p44 MAP kinase isoforms (25). This result suggests that JNK pathway is preferentially activated by cytokines. These results are in agreement with previous observations showing that the ERK module is primarily activated by mitogenic stimuli whereas JNKs are mainly activated by ceramide, cellular stress such as UV irradiation and by cytokines such as TNF- α and IL-1 β (27). However, the absence of synergy between IL-1 β and TNF- α at the level of ERKs or JNKs activation suggests that activation of MAP kinases is necessary but not sufficient to cause muscarinic m2 receptor down-regulation. These results suggest that the TNF- α and IL-1 β synergize to induce transcriptional down-regulation of M₂ muscarinic receptor, which seems to be mediated through activation of both ceramide and PKA pathways. Furthermore these results suggest that M₂ receptor expression is under the control of a cytokine network.

Platelet-derived growth factor

PDGF also down-regulated M2-receptors in HEL 299 cells and this appears to be secondary to a fall in m2 mRNA and reduced gene transcription (28). As for the other cytokines, the reduction in gene transcription was blocked by cycloheximide and therefore dependent on the synthesis of some unidentified protein. The PDGF-induced reduction in m2-receptor mRNA was not accompanied by uncoupling of the remaining receptors, unlike the situation with other cytokines so far investigated, suggesting that PDGF does not result in activation of kinases that phosphorylate muscarinic receptors. PDGF activates several signal transduction pathways with the involvement of multiple kinases. The down-regulation of m2 receptor mRNA was not inhibited by the PKC inhibitor GF 109203X, by the PKA inhibitor H-8, or by the PI-3 kinase inhibitor wortmannin. However PD 098059 completely blocked the down-regulation, indicating that MAPKK and ERKs are involved in the mechanism of down-regulation (20).

Several factors appear to influence the expression of M₂-receptors and this may have functional significance in inflammatory diseases (Table 1). Thus PKC activation, which may occur in inflammation in response to inflammatory mediators, such as bradykinin, endothelin-1 and platelet-activating factor, may inhibit the transcription of m2-receptors, resulting in reduced M₂-receptor expression. This may underlie the apparent reduction in pre-junctional M₂-receptor

Clinical interpretation

TABLE 1
Effect of various agents on M₂-receptor expression in HEL 299 cells

	Receptor density	mRNA level	Gene transcription	Coupling
Carbachol	↓	-	-	↓
β ₂ -agonists	↓	-	-	↓
Forskolin	↓	-	-	↓
PKC activation	↓	↓	↓	↓
TGF-β1	↓	↓	↓	↓
IL-1β + TNF-α	↓	↓	↓	↓
PDGF	↓	↓	↓	-

function that occurs in asthmatic patients. The cytokines TGF-β and the combination of TNF-α and IL-1β, and PDGF, all of which may be increased in asthma (24), may similarly result in a reduction in M₂-receptor expression. The mechanism by which M₂-receptor gene transcription is reduced has not yet been determined. Our studies with the protein synthesis inhibitor cyclohexamide suggest that a protein factor has to be synthesized, but the nature of this protein has not yet been determined. The promoter region of the M₂-receptor gene has not yet been sequenced, but the promoter region of the analogous M₄-receptor suggests that there may be an interaction with several transcription factors (29).

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DOES MAMMALIAN HEART CONTAIN ONLY THE M2 MUSCARINIC RECEPTOR
SUBTYPE?

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Five muscarinic acetylcholine receptor (mAChR) subtypes, m1-m5, have been cloned and sequenced to date. The question as to which mAChR subtypes exist in mammalian heart has been studied extensively and is still under considerable debate. We used the reverse transcriptase-polymerase chain reaction to amplify mRNA from adult rat ventricular myocytes, and found that these cells express mRNA for m1 and m2 mAChRs. Immunocytochemical analysis confirmed that m1 and m2, but not m3, mAChR proteins are present on the surface of these cells. Finally, the functional significance of these receptors was examined. Administration of the m1 mAChR antagonist pirenzepine inhibited the stimulatory effect of the muscarinic agonist carbachol on Ca^{2+} transients. These findings are consistent with the presence of at least two mAChR subtypes in mammalian heart, m1 and m2, and suggest that activation of m1 mAChRs is involved in the stimulatory effects of muscarinic agonists in mammalian heart.

Key Words: m1 muscarinic receptors, heart, calcium, carbachol

Stimulation of postsynaptic cardiac muscarinic acetylcholine receptors (mAChRs) is generally associated with negative inotropic and chronotropic effects, however, stimulatory responses have also been reported (1,2). These latter responses require higher concentrations of muscarinic agonists, and are often seen only after pretreatment with pertussis toxin (PTX). The inhibitory effects involve activation of m2 mAChRs which, through PTX-sensitive G_i/G_o proteins, decrease intracellular adenylyl cyclase activity, as well as activate K_{ACh} potassium channels (3). The events responsible for the stimulatory responses are not as well understood. Not only are the signal transduction pathways still undefined, but uncertainty exists as to which mAChR subtype is involved. Indeed, considerable debate remains as to whether m2 mAChRs are the only muscarinic receptor subtype in mammalian heart.

Of the five mAChR subtypes which have been cloned and sequenced, Northern blot analyses of rat and guinea pig heart detected only m2 mAChR mRNA (4,5). Results from radioligand binding studies, however, were not as conclusive. While some studies suggested the existence of a single mAChR subtype (6), others found evidence for a second subtype of muscarinic receptor (7,8). Further evidence for an additional type of mAChR came from

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experiments performed in Purkinje fibers isolated from young dogs. In these experiments, the muscarinic receptor agonist acetylcholine increased automaticity via mAChRs which were sensitive to pirenzepine, suggesting the involvement of m1 mAChRs (9). In the studies presented here, we investigate the possibility that mAChR subtypes other than m2 are present in mammalian heart. We show that m1 mRNA and receptor are indeed expressed in these cells. Furthermore, we show that m1 mAChRs are involved in the stimulatory responses in heart.

Methods

Cell Isolation and Purification

Myocytes were isolated from adult male Sprague Dawley rats (250-300g) according to methods previously described by our laboratory (10). Briefly, isolated hearts were perfused with Ca^{2+} -free Joklik's tissue culture media (GIBCO) until the hearts were cleansed of blood. Next, a Joklik's solution containing 0.5 mg/ml Type II collagenase (Worthington Biochemical), 50 $\mu\text{mol/L}$ CaCl_2 , and 0.1% BSA was perfused through the heart for ≈ 30 min, after which the ventricles were minced and shaken in the collagenase solution to dissociate the ventricular myocytes. Finally, dissociated cells were collected by centrifugation for 5 min at 34g.

Reverse Transcription-Polymerase Chain Reaction

Isolation of cellular RNA and subsequent RT-PCR were performed as previously described by our laboratory (11). The primers used to direct synthesis of the five known rat mAChRs have been previously described in detail (11). For single cell RT-PCR, individual myocytes were collected using negative pressure through a glass pipette having a tip diameter of 2-3 μm . First strand cDNA synthesis and subsequent PCR were carried out according to the protocol of Grigorenko and Yeh (12).

Immunofluorescence Studies

Isolated myocytes were fixed with 1% paraformaldehyde, and permeabilized with 0.3% Triton X-100. Cells were first incubated with goat serum to block non-specific binding sites, and then with affinity-purified rabbit polyclonal antibodies targeted against m1, m2 and m3 mAChRs (13). Excess primary antibody was washed out, and cells were exposed to a fluorescein-labeled goat anti-rabbit antibody (Gibco). After removal of excess secondary antibody, cells were mounted on glass coverslips with Mowiol 4-88 (Calbiochem). Digital imaging was used to visualize the fluorescence labeled cells using an excitation wavelength of 490 nm and acquiring images at 530 nm. Details of the digital imaging technique have been published previously (14).

Calcium Transient Recordings

The detailed procedure for recording Ca^{2+} transients in adult ventricular myocytes has been described in our previous publications (15,16). Briefly, isolated myocytes were loaded with 1 $\mu\text{mol/L}$ of the fluorescent Ca^{2+} indicator fura 2-AM (Molecular Probes) and the coverslip containing dye-loaded cells was mounted in a tissue chamber (Bellco) on the stage of a Nikon Diaphot inverted microscope equipped for epifluorescence. The cells were stimulated at a frequency of 1 Hz with an S8 Grass stimulator, and fluorescence was measured at 340 and 380 nm.

Results

Identification of mAChR Subtypes in Rat Heart

In an effort to carefully examine the mAChR subtypes present in rat heart, we performed RT-PCR on total RNA from ventricular myocytes isolated from adult rat heart. Figure 1 shows that both m1 and m2 mAChR message was amplified from total mRNA (lanes 2 and 4), whereas

m3 (lane 6), m4 (lane 7) and m5 (lane 8) mRNA was not. These results were consistent with the presence of m1 and m2 mAChR mRNA in these cells, and the identity of these products was validated by restriction mapping and southern blot analysis (data not shown).

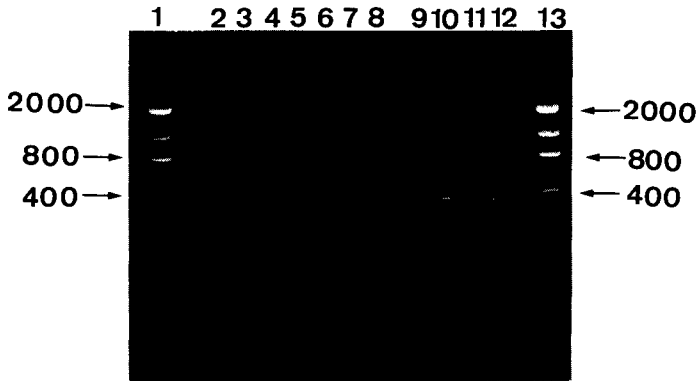


Fig. 1

Gel electrophoresis of RT-PCR products amplified from total RNA and single myocytes. RT-PCR was carried out on total RNA from isolated ventricular myocytes (lanes 2-8) and RNA isolated from single myocytes (lanes 9-12). DNA products were electrophoresed on 1.2% agarose gel and visualized by staining with 0.5mg/ml ethidium bromide. Lanes 2 and 4 show amplification from total RNA of message for m1 and m2 mAChR respectively. No message was amplified for m3, m4 and m5 mAChRs (lanes 6-8, respectively). Single cell PCR (lanes 9-12) shows amplification of m1 and m2 mAChR message (lanes 12 and 10, respectively). No amplification of m1 (lanes 3 and 11) or m2 (lanes 5 and 9) mAChR mRNA was obtained when reverse transcriptase was omitted from the RT reaction. (Adapted from reference 11)

A potential problem with interpretation of these results stems from the inability to obtain a preparation of pure myocytes. Although evaluation of our preparation under high power magnification (400X) suggests that it is pure, contamination with other cell types (e.g. fibroblasts, smooth muscle cells, nerve endings) could not be ruled out. Indeed, if we culture the isolated cells for 16-24 hours, fibroblasts become detectable under the microscope. Since the technique of RT-PCR is sensitive enough to amplify even the smallest amount of contaminating RNA, we felt that any possible contribution from contaminating cell types had to be eliminated. Thus, we decided to repeat these experiments using RNA that was isolated from single myocytes. Using this protocol, we could be assured of collecting and amplifying RNA from myocytes only, thereby ruling out the possibility of amplifying cDNA from other cell types. Under these conditions, m1 and m2 mAChR mRNA was amplified (lanes 12 and 10, respectively), suggesting that the PCR products obtained in lanes 2 and 4 were indeed amplified from myocytes. Finally, to confirm that these PCR products were amplified from cDNA and not genomic DNA, control RT reactions were performed in the absence of reverse transcriptase. In these instances, no DNA for m1 or m2 mAChRs was amplified (lanes 3 and 5, respectively), confirming that the cDNA and not the genomic DNA had indeed been amplified. Taken together, these data are consistent with the presence of m1 and m2 mAChRs in rat heart.

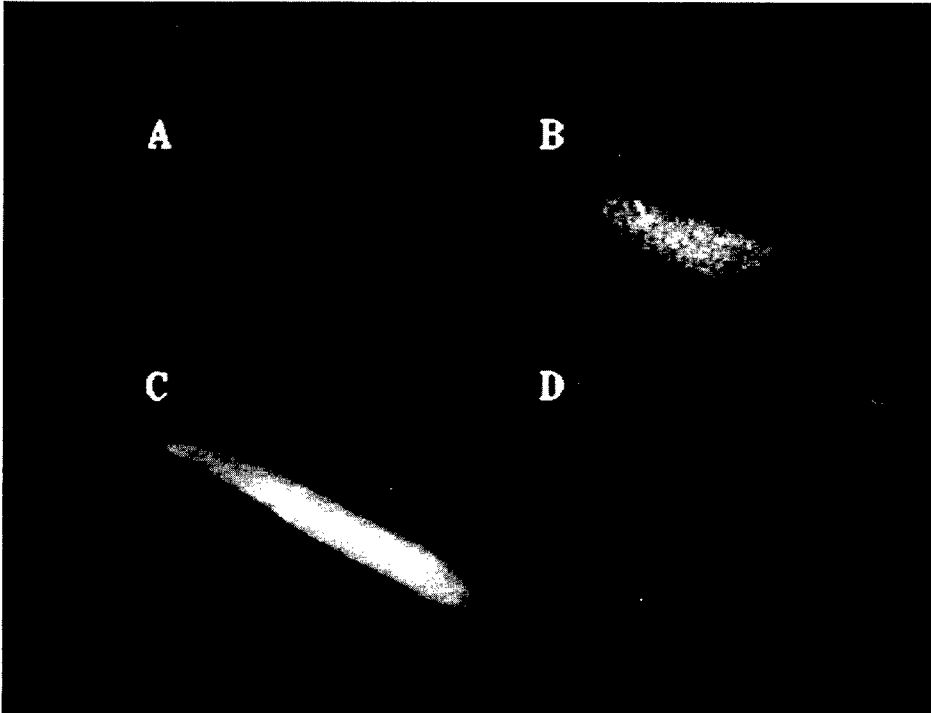


Fig.2

Immunofluorescent detection of mAChR subtypes. Subtype specific antibodies detected m1 (panel B) and m2 (panel C) mAChRs on isolated ventricular myocytes. No staining was seen with m3 antibody (panel D), or in the absence of primary antibody (panel A). (Adapted from reference 11)

Immunofluorescent Detection of mAChR Subtypes

Although the presence of m1 mAChR RNA in the rat ventricular myocytes strongly suggests that rat heart possesses m1 mAChRs, further evidence was required to show that this mRNA was indeed translated into a protein which became expressed on the cell surface. Therefore, adult ventricular myocytes were incubated with subtype-specific mAChR antibodies (m1-m3), and the distribution of these antibodies was visualized using a fluorescein-tagged second antibody. Figure 2 shows diffuse fluorescent staining in the cells incubated with m1 (panel B) and m2 (panel C) mAChR antibodies, but not in cells incubated with antibody to the m3 mAChR (panel D). Furthermore, no fluorescence was detected in cells incubated with no primary antibody (panel A). The fluorescence in cells treated with antibody directed against m2 mAChR was much greater than that in cells incubated with m1 mAChR antibody, consistent with m2 mAChRs being the predominant mAChR in adult rat ventricular myocytes.

Physiological Significance of m1 mAChR Activation

Finally, the physiological relevance of the m1 mAChR expression was investigated. We have previously shown that high concentrations of muscarinic agonists (e.g. carbachol; CCh) can increase basal $[Ca^{2+}]_i$ in isolated rat ventricular myocytes (17). In an effort to determine whether m1 mAChRs are involved in this stimulatory response, we examined whether blockade of m1 mAChRs with pirenzepine could inhibit this effect. In PTX pretreated cells (100 ng/ml for 16-18 hours), 300 μ mol/L CCh increased basal $[Ca^{2+}]_i$ from 96 ± 7 to 118 ± 8 nmol/L, and peak Ca^{2+} was elevated from 519 ± 32 to 640 ± 36 nmol/L ($n=8$). As evident in the representative experiment shown in Fig. 3A, both of these effects were reversed by 10 nmol/L pirenzepine. The m2 selective antagonist methoctramine (100nmol/L) had no effect on these responses (Fig. 3B). Taken together, our results support a role for m1 mAChR activation in the increase of basal Ca^{2+} in the mammalian heart.

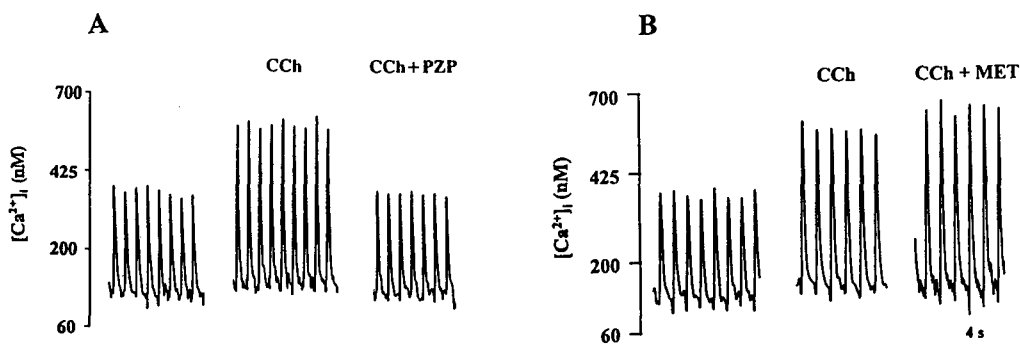


Fig.3

Effect of CCh on Ca^{2+} transients in PTX-treated heart cells. Ca^{2+} transients were elicited at 1 Hz, and the effect of 300 μ mol/L CCh was assessed in the absence and presence of 10 nmol/L pirenzepine (panel A) or 100nmol/L methoctramine (panel B) by measuring fluorescence emitted by the Ca^{2+} indicator fura-2 AM.

Discussion

The findings of this study support the existence of multiple mAChR subtypes in adult rat heart. Furthermore, evidence is provided which supports a role for activation of these receptors in the CCh-induced stimulatory responses in cardiac cells. The mechanisms by which mAChR activation produces both inhibitory and stimulatory effects on mammalian heart are still unresolved. More specifically, controversy exists regarding the mAChR subtype(s) involved in these pathways. While activation of m2 mAChRs is the established pathway responsible for initiation of the inhibitory effects, debate remains concerning the stimulatory effects. One hypothesis is that the m2 mAChR not only initiates the inhibitory responses, but, the stimulatory responses as well. This is supported by radioligand studies (6), as well as the inability to detect more than one mAChR subtype by northern blot analysis (4,5). In this scenario, the m2 receptor couples not only to a PTX-sensitive G_i/G_o protein, but also, with less efficiency, to a separate second messenger system. This latter system would involve either a PTX-insensitive G-protein

such as G_q or, a G-protein independent system. Indeed, muscarinic agonists have been reported to increase Na^+ influx through muscarinic receptor operated channels (18) in a guanine nucleotide independent manner (19). Furthermore, it has been suggested that activation of this inward Na^+ current activates Na^+/Ca^{2+} exchange, producing a positive inotropic effect (18).

Alternatively, a second subtype of mAChR could exist whose activation initiates the stimulatory responses. In contrast to the binding studies mentioned above, similar experiments done in other laboratories suggest the presence of a second population of mAChRs (7,8). RT-PCR experiments have amplified m1 mAChR mRNA from neonatal and adult rat ventricular myocytes (11,20), as well as from guinea pig ventricular myocytes (5). Notably, when northern blot analysis was done on mRNA isolated from the guinea pig ventricular myocytes, only m2 mAChR mRNA was detected (5). This suggests that the northern blot technique is not sensitive enough to detect relatively low levels of mRNA. In addition to the possible increase in Ca^{2+} via m2 mAChR activation mentioned above, two mechanisms have been proposed to account for m1 mAChR mediated stimulatory effects of muscarinic agonists. The first involves stimulation of membrane phospholipid hydrolysis (21) and subsequent activation of PKC. The second possible mechanism involves direct activation of L-type Ca^{2+} currents by m1 mAChRs (5). Which of these three signal transduction mechanisms contribute to the positive inotropic and chronotropic effects of muscarinic agonist remains to be determined.

Interestingly, in the RT-PCR studies done by our laboratory on mRNA from neonatal rat ventricular myocytes, we amplified mRNA for m1, m2 and m4 mAChRs (20). The reason for the presence of this additional mAChR subtype in the neonatal cells is not readily apparent, however, one possibility is that the m4 mAChR plays a significant role in early developmental stages of rat heart. Clearly, further work is required to determine a role for the m4 mAChR in neonatal rat heart.

In conclusion, we have shown that m1 mAChRs coexist with m2 mAChRs in adult rat heart, and that the former are indeed involved in the stimulatory responses which have been documented in response to activation of mAChRs with high concentrations of agonist. While the physiological significance of these responses is not clear, it has been suggested that they may exist in an autoregulatory capacity to counteract any excessive inhibition of heartbeat by the vagus nerve (22). Indeed, other receptor systems involved in maintaining cardiac function (e.g. adenosine and α -adrenergic) are also able to produce opposing effects (23,24).

Acknowledgments

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MUSCARINIC ACETYLCHOLINE RECEPTOR SUBTYPE, m2: DIVERSE FUNCTIONAL IMPLICATIONS OF DIFFERENTIAL SYNAPTIC LOCALIZATION

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Summary

The muscarinic acetylcholine receptor (mAChR) molecular subtype, m2, has been postulated to be the presynaptic cholinergic autoreceptor in many brain regions. However, due to a lack of subtype-specific pharmacological agents, conclusive evidence for m2 as an autoreceptor remains elusive. The development of subtype-specific antibodies has enabled extensive characterization of the synaptic localization of the m2 subtype. Specifically, double-labeling immunocytochemistry with m2 antibodies and antibodies to the vesicular acetylcholine transporter (VACHT), a novel specific marker of cholinergic terminals, in the striatum has allowed the first direct anatomical evidence of m2 localization in cholinergic terminals. Additionally, other anatomical studies in striatum and the septohippocampal pathway have revealed that this subtype is also expressed presynaptically in non-cholinergic terminals, and is postsynaptically expressed in both cholinergic and non-cholinergic neurons. The implications of these data for understanding the functional roles of this subtype are discussed.

Key Words: presynaptic autoreceptor, presynaptic heteroreceptor, hippocampus, striatum, m2 muscarinic acetylcholine receptor

Presynaptic muscarinic autoreceptors that inhibit the release of acetylcholine (ACh) play a key role in the modulation of cholinergic transmission in the peripheral and central nervous systems. Many physiological studies have confirmed the existence of such a receptor using slice preparations, synaptosomal preparations, and other approaches (1, 2). For example, cholinergic agonist actions at presynaptic autoreceptors decrease synaptic potentials in striatal neurons in slice preparations (3, 4). Muscarinic autoreceptors have also been identified by functional studies in the basal forebrain, cortex, hippocampus and striatum (1, 2).

Muscarinic autoreceptors are of therapeutic interest in the search for drugs that modulate physiologically released stores of ACh. For example, drugs selective for the autoreceptor have been suggested as a treatment for Alzheimer's disease, a disease in which basal forebrain cholinergic neurons are lost (5). It has been suggested that the loss of cholinergic input to the hippocampus and cortex from the basal forebrain contributes to the dementia syndrome (5). While investigators search for ways to stimulate postsynaptic mAChRs with cholinomimetics in order to replace decreased cholinergic transmission (6, 7), antagonizing the autoreceptor may allow for increased ACh release from surviving cholinergic cells (8). However, in order to target drugs appropriately to achieve such goals requires knowledge of the molecular identity of the autoreceptor.

Pharmacological studies have identified autoreceptors in many brain regions as belonging to the M₂ pharmacological class of receptors (2, 9-15). While many investigators have extrapolated from these findings and have concluded that the molecular subtype, m2, is the autoreceptor, direct evidence for this is lacking. Characteristic M₂-selective agents have almost equal affinities for both the m2 and m4 molecular subtypes (16, 17), and somewhat lower affinities for m3 (16). Moreover, the pharmacological characterization has been controversial, with studies variously labeling the hippocampal autoreceptor as M₂, M₃ or M₄ (2, 9).

Anatomical approaches have yielded equally controversial data. Autoradiographic studies have shown that M₂ receptor binding sites are high in regions with cholinergic cells (18, 19). *In situ* hybridization and immunocytochemical studies have afforded greater molecular specificity and cellular resolution to identify m2 mRNA and protein in cholinergic as well as non-cholinergic cells in many brain regions (18, 20). While mRNA and protein expression in the cell bodies of cholinergic cells is requisite for presynaptic expression on cholinergic terminals, it does not necessarily mean that the m2 receptor is actually localized and functional in terminals. In fact, postsynaptic m2 receptors have been identified in many neuronal populations in cortex, medial septum and striatum (20-22). In addition, many of the m2 receptors in the basal forebrain are presynaptic rather than within cholinergic cell bodies (20). Electron microscopic immunocytochemistry has also demonstrated a presynaptic localization of the m2 protein in many brain regions, but the cholinergic identity of these terminals has not yet been directly confirmed (20, 21).

Here we review recent studies from our laboratory, and provide new information addressing localization of the m2 receptor protein in the striatum and septohippocampal projection, two key central cholinergic systems in the mammalian forebrain. The data presented here are foundational to understanding m2's functional roles as a cholinergic autoreceptor, as well as a heteroreceptor regulating the release of other neurotransmitters, and a postsynaptic effector.

Methods

Sprague-Dawley rats 250-300 g were deeply anesthetized with 4% chloral hydrate, then perfused transcardially with a phosphate buffered solution of 3% paraformaldehyde and 0.15 % or 0.2% glutaraldehyde for 12-13 minutes (260-290 ml). The rats were then refrigerated for 1-2 hours, after which their brains were removed and 40-50 μ m sections of the striatum were obtained on a vibratome (Oxford). Sections were subsequently preblocked with 4% normal goat serum (NGS) in tris buffered saline (TBS) (50 mM tris, 1.5 % NaCl, pH 7.2) and then incubated in primary m2 monoclonal antibody (1:500 from concentrated cell culture medium) (20) and VACHT polyclonal affinity purified antibody (1 μ g/ml) (23) in TBS containing 2% normal goat serum for at least 48 hours. Control sections were treated identically, except they received only one of the two primary antibodies or no primary antibody. After several rinses in TBS, the sections were incubated in the following secondary antibodies for 24 hours: goat anti-rat (for monoclonal m2) and gold conjugated goat anti-rabbit (for polyclonal VAT). The m2 monoclonal immunoreactivity was amplified with rat peroxidase anti-peroxidase (PAP) and developed in diaminobenzidine (DAB, Sigma) containing 0.5% hydrogen peroxide, rinsed in TBS followed by a phosphate buffer rinse and then post-fixed overnight in 4% glutaraldehyde in phosphate buffer. Sections were then rinsed thoroughly in 0.1M phosphate buffer (pH 7.6), processed with a silver enhancement protocol as described in (24) and postfixed in 1% osmium tetroxide in phosphate buffer for one hour. Sections were then dehydrated through a graded ethanol series, followed by propylene oxide, infiltrated and embedded in Eponate 12 (Ted Pella, Inc.) between glass slides coated with releasing agent. Striatal sections were dissected and placed on precured stubs for thin sectioning. Silver and silver-gold sections were obtained using a diamond knife on a Reichert Ultracut S (Leica) ultramicrotome. Sections were retrieved on 200 mesh hexagonal grids, viewed and photographed on a JEOL 100 C electron microscope. Other sections were incubated in the following secondary antibodies: goat anti-rabbit (for polyclonal VACHT) and gold conjugated goat anti-rat (for monoclonal m2). In this case VACHT was developed with DAB and m2 was silver intensified and the sections were processed as described above.

Results and Discussion

m2 as an autoreceptor

One forebrain cholinergic system resides in the striatum, a basal ganglia structure that plays an important role in motor control. The main source of ACh in the striatum is provided endogenously by large interneurons. Immunocytochemically these neurons are easily identifiable by immunoreactivity for cholinergic markers such as choline acetyltransferase (ChAT) and the VAcHT (23). Pharmacological studies have identified a muscarinic autoreceptor in the striatum that is responsive to M₂ selective ligands (10 443, 25, 26). Also, recent studies on striatal cholinergic interneurons have shown that N- and P- type calcium currents are inhibited by M₂ receptors (27), providing a mechanism by which receptors of this subclass regulate transmitter release.

Anatomical studies in the striatum indicate that the m2 subtype is uniquely expressed in cholinergic neurons (20). For example, m2 immunoreactivity shows nearly complete co-localization with ChAT immunoreactivity in large striatal cell bodies (20). At the electron microscopic level, m2 has been also identified in cell bodies of cholinergic interneurons (21). In addition, m2 is localized in axon terminals making symmetric contacts (21), similar morphologically to cholinergic terminals. However, since terminals containing other transmitters also make symmetric contacts, these data do not conclusively identify m2 in cholinergic terminals.

Recently, antibodies to VAcHT, a novel and specific cholinergic marker, were developed by Gilmor et al. (1996). VAcHT is uniquely expressed in cholinergic neurons throughout the brain, including striatal interneurons. VAcHT is concentrated in cholinergic terminals where it is localized in synaptic vesicles, consistent with its role in packaging vesicular stores of ACh. We used double labeling immunocytochemical techniques at the electron microscopic level to determine if m2 and VAcHT are co-localized in the same terminals. As shown in Fig. 1, m2 was visualized with rat monoclonal antibodies using immunoperoxidase techniques that produce a diffuse reaction product, and VAcHT was localized using rabbit polyclonal antibodies and rabbit-specific immunogold labeled secondary antibodies that were silver enhanced to produce highly electron dense deposits. The results show m2 receptor co-localization with VAcHT in cholinergic terminals, and provide the first direct anatomical identification of m2 as a presynaptic autoreceptor.

In contrast to the striatum where m2 is expressed in virtually all cholinergic neurons, but not in non-cholinergic neurons, m2 localization in other cholinergic projections, such as the septohippocampal pathway, appears more complex. The basal forebrain provides an extensive cholinergic innervation that modulates many areas of cortex and hippocampus (28, 29). The septohippocampal component of this system arises from cholinergic neurons in the medial septum/diagonal band complex, and has been shown to regulate memory related phenomena such as the theta rhythm (30-32), long term potentiation and long term depression (33).

Pharmacologically, the presynaptic receptor that regulates acetylcholine release in the basal forebrain and the hippocampus belongs to the M₂ subclass (9, 11, 13, 15, 34-37). Both *in situ* hybridization (18, 38, 39) and antibody studies (20, 40) have demonstrated that the m2 subtype is the most abundant mAChR in the basal forebrain. Cholinergic neurons (as identified by the presence of ChAT) in the MS/DB express m2 mRNA and are immunoreactive for m2 protein (20), confirming the localization of m2 in cholinergic cells of the basal forebrain (Fig. 2). Since the basal forebrain provides the predominant cholinergic input to cortex and hippocampus, it was assumed that m2 expression in basal forebrain neurons was indicative of synthesis of the receptor prior to transport to presynaptic terminals in the projection areas. This possibility is supported by lesions of the septohippocampal pathway which result in about a 25% decrease in m2 protein in the hippocampus as detected by immunoprecipitation (41, 42). Electron microscopic immunocytochemical studies have confirmed that m2 is localized in axon terminals in rat hippocampus (43) and monkey neocortex (22). Although these m2-positive terminals are morphologically similar to cholinergic terminals, i.e., with small round clear vesicles and in symmetric synaptic contacts, many other neurotransmitter containing terminals have the same appearance. Thus, co-localization of m2 with markers selective for cholinergic terminals, such as VAcHT, will be necessary to conclusively identify m2 as a presynaptic autoreceptor on the septohippocampal pathway and other parts of the basal forebrain projection systems.

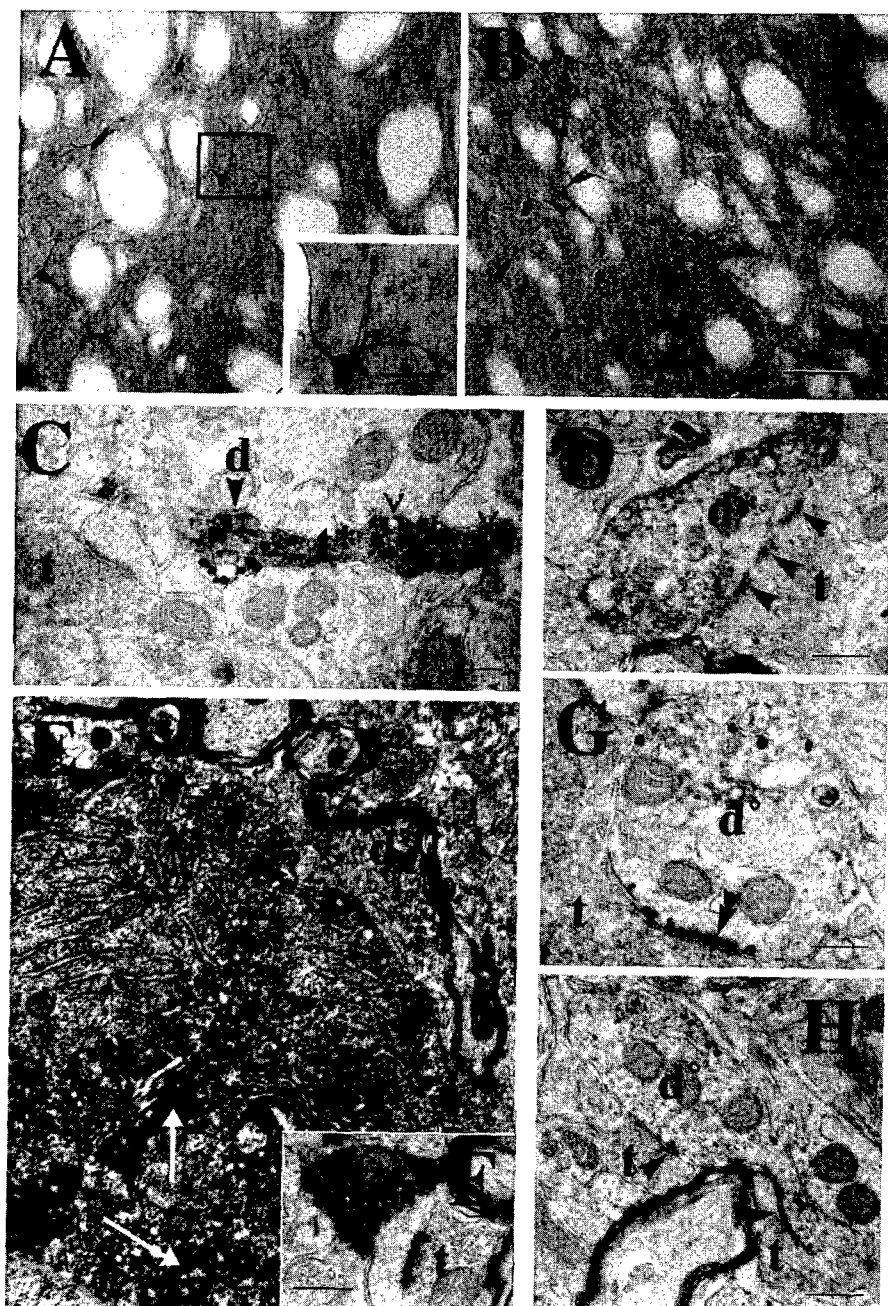


Figure 1

Light and electron microscopic localization of m2 and VAcHT in the rat neostriatum. **A:** light level m2 (DAB) immunoreactivity with inset showing a single, high power m2 immunoreactive

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neuron. **B:** light level VACHT (DAB) immunoreactivity. **Note that the m2 acetylcholine receptor (AChR) and the vesicular acetylcholine transporter (VACHT) show similar cellular and neuropil staining in the striatum.** **C:** axon terminals double-labeled (t^*) with m2 (DAB: diffuse reaction product) and VACHT (gold: dark black particles). Some of the terminals are seen making symmetric contacts (single arrowheads) onto unlabeled dendrites (d). Note that the gold particles are associated with synaptic vesicles (open arrowheads) as would be expected for the VACHT protein. **Co-localization of m2 and VACHT in this terminal demonstrates the presence of the m2 receptor in a cholinergic terminal where it is in position to act as an autoreceptor.** Also, note the presence of an unlabeled axon terminal (t). **D:** large diameter dendrite double labeled (d^*) with m2 (DAB) and VACHT (gold). The dendrite is receiving synaptic contact (arrowheads) from an unlabeled axon terminal (t). **This demonstrates that m2 is in a position to act as a postsynaptic receptor.** **E:** partial cell body (c^*) with extensive m2 (gold) and VACHT (DAB) labeling. m2 gold particles are seen in the ER and golgi (open arrowheads) reflecting biosynthetic pathways, as well as plasma membrane gold particles (arrows) reflecting cell-surface pools of the m2 receptor. Diffuse VACHT immunoreactivity (white arrows) fills the cytoplasm. **F:** axon terminal double labeled (t^*) with m2 (gold) and VACHT (DAB), near an unlabeled axon terminal (t). **G-H:** dendrites single labeled (d^0) with m2 (gold). Note the asymmetric (double arrowhead) and symmetric (arrowhead) contacts onto these dendrites. **These dendrites show that m2 mAChRs are associated with the dendritic plasma membrane, and therefore, are in a position to act as postsynaptic receptors.** **Scale bars:** A, B = 100 μ m; inset = 50 μ m; C = 0.30 μ m; D = 0.43 μ m; E = 0.68 μ m; F = 0.37 μ m; G, H = 0.30 μ m.

m2 as a presynaptic heteroreceptor

Presynaptic mAChRs in the hippocampus not only inhibit the release of ACh, they also regulate the release of GABA, glutamate and aspartate. Sources of these neurotransmitters lie both intrinsic and extrinsic to the hippocampal formation, with modulation of their release mediated by M_2 and M_2 -like mAChRs (44-48). Again, since the pharmacological class of M_2 receptors includes both m2 and m4 molecular subtypes (16, 17), it is important to examine the anatomical evidence in order to investigate if m2 may also function presynaptically as a heteroreceptor in the hippocampus. In fact, the anatomical data suggest that it does.

Lesion studies indicate that only a small percentage of hippocampal m2 is associated with the cholinergic septohippocampal projection. Fimbria-fornix lesions as well as selective cholinergic lesions only result in a small decrease in hippocampal m2 expression as evaluated by immunoprecipitation and immunocytochemistry (41), indicating that the majority of m2 in the hippocampus is not associated with cholinergic fibers from the septum. Electron microscopic studies of the CA1 regions and the dentate gyrus indicate that m2 is localized presynaptically in non-cholinergic terminals throughout the hippocampal formation (49) (Fig. 2). Many of these m2 positive terminals contain GABA (49). Lesion studies of the hippocampal associational (50) and perforant pathways (Rouse and Levey, unpublished observations) indicate that m2 is presynaptically localized in these aspartatergic (51-53) and glutamatergic pathways respectively, suggesting a heteroreceptor role for m2 in the modulation of excitatory amino acid transmitter release.

m2 as a postsynaptic receptor

It is clear that the actions that are mediated by the m2 receptor are not solely presynaptic. In the striatum and the medial/septum diagonal band m2 is found in numerous cell bodies and dendrites at both the light and electron microscopic levels (20) (Fig. 1). In the basal forebrain, m2 is in a postsynaptic position to regulate the neurons via ACh release from axon collaterals (54) and from cholinergic mesopontine tegmental neurons which terminate in this region (55). Ionophoretically applied ACh excites 70% of septohippocampal neurons in an atropine sensitive manner (56). In addition, carbachol elicits two opposing responses on medial septum/diagonal band neurons (57), a hyperpolarizing outward current that appears to be mediated by the M_2 subclass of mAChRs, and a depolarizing inward current that appears to be mediated by either the M_1 or M_2 subclasses (58).

m2 receptor locations:

- 1 Postsynaptic receptor
- 2 Presynaptic autoreceptor
- 3 Presynaptic heteroreceptor

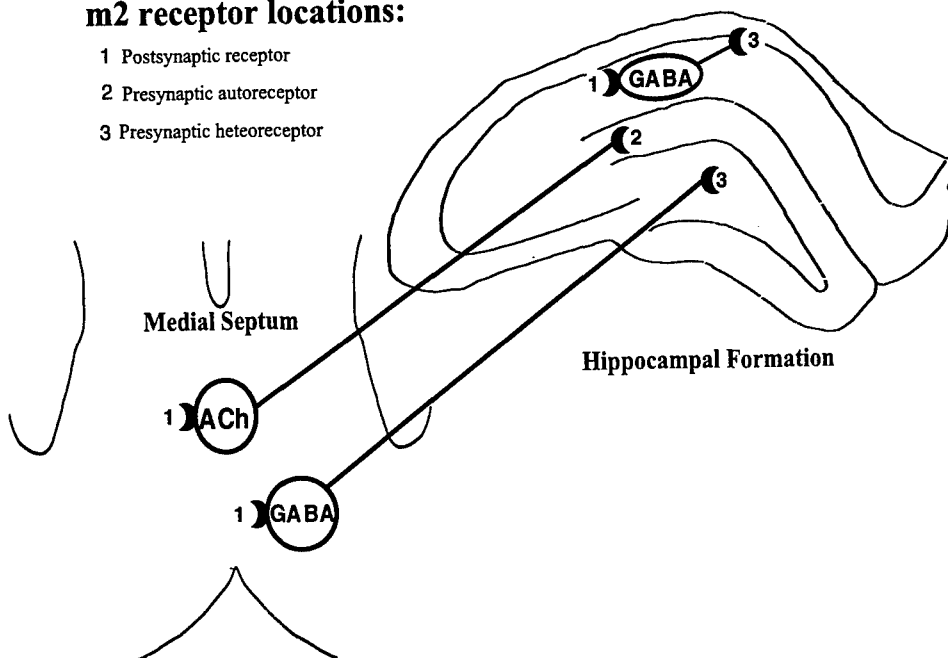


Figure 2

A schematic representation of the synaptic localization of m2 in the septohippocampal pathway. **Postsynaptic receptor (1):** The m2 subtype has been identified in cell bodies of both cholinergic and non-cholinergic septohippocampal projection neurons. In the hippocampus, m2 has been identified in the somata and dendrites of GABAergic interneurons. **Presynaptic autoreceptor (2):** Anatomical studies indicate that in the hippocampus, m2 may be expressed in terminals of cholinergic septohippocampal cells. **Presynaptic heteroreceptor (3):** In the hippocampus, the m2 subtype has been identified in GABAergic terminals most likely from GABAergic septohippocampal cells and GABAergic interneurons.

M₂-mediated postsynaptic responses, such as the blockade of the M-current, a voltage and time dependent potassium current (59), are found in the hippocampus as well. In the hippocampus, at the subcellular level, m2 is found in numerous dendritic profiles and cell somata confirming its post-synaptic location in this structure. In the CA1 region m2 immunoreactive interneurons (Fig. 2) have been identified at the border of the stratum oriens and the alveus (41). These layers also contain many m2 immunoreactive dendritic profiles (Edmunds and Levey, unpublished observations). Other m2 immunoreactive interneurons are found in CA1, CA3 and the hilus of the dentate gyrus scattered throughout many layers (41). Many of these are GABAergic interneurons (49), suggesting that ACh modulates the activity of inhibitory interneuron populations via postsynaptic m2 receptors. Physiological studies have shown that carbachol increases inhibitory postsynaptic potentials in pyramidal cells potentially through an mAChR mediated excitation of inhibitory neurons (48, 60)

Conclusions

Immunocytochemical electron microscopic studies reveal a diversity of synaptic localizations of the m2 receptor protein. Localization of the m2 receptor in VAcHT immunoreactive terminals places this subtype in a position to regulate cholinergic transmission as a presynaptic autoreceptor. In addition, m2 localization in non-cholinergic terminals in hippocampus suggests this subtype regulates the major excitatory and inhibitory neurotransmitters as presynaptic heteroreceptors. Moreover, m2 is situated to regulate postsynaptic excitability of cells in the basal forebrain and interneurons of the hippocampus and striatum. Understanding the anatomical and functional properties of m2 provides insight to the complex network of muscarinic receptors and their modulatory impact on a vast number of central nervous system circuits.

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MUSCARINIC M₃ RECEPTOR COUPLING AND REGULATION

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Summary

Current concepts regarding the regulation and coupling of muscarinic m₃ receptors to G-proteins and various effectors are discussed. The last few years have provided much evidence that although muscarinic m₁, m₃ and m₅ subtypes couple predominantly via pertussis toxin-insensitive G-proteins (Gq/11) to activate phosphoinositidase C (PIC), interactions with other G-proteins (Gi, Go, Gs) can be readily observed in cells expressing recombinant muscarinic receptors even at relatively low levels. The significance of this diversity and the potential for agonist "trafficking" could open up opportunities for novel approaches to selective agonist action. Finally, mechanisms underlying the regulation of muscarinic m₃ coupling through Gq/11 to PIC are discussed. In particular, our recent studies on precursor lipid depletion, whether regulation is receptor or cell specific and the identification and rôle of receptor kinases are briefly reviewed.

Key Words: M₃ receptors, G proteins, muscarinic receptor regulation

Evidence from molecular cloning indicates that there are separate human genes that encode five muscarinic receptor subtypes and their expression in model cells following cDNA transfection has revealed receptors with properties that, in general, are consistent with the pharmacological characteristics of particular muscarinic receptors in isolated mammalian tissues [1,2]. Furthermore, the lack of sufficiently selective ligands for muscarinic subtypes combined with the co-expression of more than one subtype in most tissues, has resulted in the increased use of transfected cell lines expressing a single receptor. However, it is not always clear whether host cell lines are representative, in terms of their G-protein and effector repertoire, of the cellular environment that the receptor experiences *in vivo*. Nevertheless, the expression of cloned receptors in cell lines does provide pure populations in an identical cellular environment so the factors can be identified that allow different subtypes to be operationally distinguished.

Over the last six years we have extensively investigated the coupling and particularly the regulation of human recombinant muscarinic receptors expressed in cell lines. Here we wish to summarise some of the more recent developments in our understanding of G-protein linked receptor coupling and to highlight some of our thinking on the regulation of m₃ and m₁ receptor coupling to phosphoinositidase C and Ca²⁺ mobilisation in cells.

Promiscuous Muscarinic M₃ Receptor Coupling and Ligand Trafficking

The m₃ muscarinic receptor is generally accepted to predominantly couple to the membrane bound enzyme phosphoinositidase C β (PIC β) via the pertussis toxin insensitive Gq/11 G-proteins [1,2]. Agonist activation of the m₃ muscarinic receptor therefore increases PIC β activity and accelerates the rate of phosphatidylinositol (4,5) biphosphate (PIP₂) hydrolysis. The breakdown of PIP₂ leads to the formation of inositol 1,4,5-trisphosphate (Ins(1,4,5)P₃) and diacylglycerol (DAG) and these breakdown products act as second messengers by mobilising Ca²⁺ from intracellular stores and activating protein kinase(s) C (PKC) respectively [3,4]. However, over the last few years it has become clear that seven transmembrane spanning (7TM) receptors, including muscarinic receptors, are promiscuous and can form interactions with multiple G-proteins and effectors [5,6,7,8]. Therefore, agonist potency not only depends upon receptor density but can also be affected by variations in the amount and type of G-protein which are available to interact with the receptor in a particular tissue and the affinity of the receptor for those G-proteins. Furthermore, variations in effector enzymes or channels present in the tissue will also change the final response to agonist [see 9,10]. Kenakin and colleagues [9,11] have discussed the theoretical outcome of receptor G-protein promiscuity and others have emphasised the stoichiometry of receptors and G-proteins and particularly the access between these proteins within microdomains of intact cells [12].

While there is substantial evidence for the presence of receptor promiscuity in recombinant systems, the likelihood of multiple interactions of a single receptor to various G-proteins must increase with high concentrations of reactants. The question of its physiological significance is much debated though it is the opinion of the authors that there is at least persuasive evidence from studies with the muscarinic m₃ receptor. "Physiological" levels of recombinant m₃ receptor expression in host cells clearly exhibit interactions with both Gi type as well as Gq/11 proteins. Agonist-GTP interactions at m₃ binding sites have been shown to be pertussis toxin-insensitive as has agonist stimulated GTP γ S binding to membrane preparations [13]. Furthermore, direct evidence for m₃ receptor interaction with Gi proteins has been revealed using subtype-specific immunoprecipitation of G α subunits photolabelled with [α -³²P] GTP azidoanilide [14]. Promiscuity of recombinant muscarinic receptor subtype coupling to multiple G-proteins is maintained in intact cells [15] and there is indirect evidence for multiple signal transduction in parotid gland in which a homogenous m₃ population is claimed [16].

Overall, therefore there is increasing evidence for multiple interactions between receptors and different G-proteins and the implications to signal transduction are just being realised. In particular, the release of $\beta\gamma$ subunits from Gi proteins may provide an unsuspected diversity of signalling in addition to activation of PIC via Gq/11 mediated by muscarinic (m₁, m₃, m₅) subtypes. Very recent studies from our laboratory [17] (and unpublished) have revealed that inactivation of Gi proteins with pertussis toxin greatly enhances phosphoinositide hydrolysis mediated by recombinant metabotropic glutamate (mGlu1 α) receptors via Gq/11 in BHK cells. Whether this can be established for m₃ muscarinic receptors is not known, although these observations and evidence of $\beta\gamma$ subunit mediated inhibition of PIC β 1 [18] suggests that dual regulation by G-proteins of this fundamental signalling pathway requires further consideration.

Although it is generally agreed that receptors can be promiscuous with G-proteins, it is currently of much interest to pharmacologists whether all agonists can activate multiple G-proteins or whether different agonists can direct or 'traffic' receptors to different transducing proteins. The implications of this 'agonist trafficking' concept are potentially enormous to

pharmacology with the concentration and intrinsic efficacy of an agonist (as accepted in classical models) not only determining the extent but also the 'quality' of a cellular response by influencing different G-protein mediated signalling cascades. There are now several examples of such trafficking [9,10] perhaps including muscarinic receptor subtypes. Recent studies for example have shown that agonists such as carbachol preferentially couple m₁ rather than m₃ receptors to Gs and the stimulation of adenylate cyclase [15,19]. Furthermore, several partial agonists such as pilocarpine and McN-A-343 display selective activation of m₁ over m₃ receptors in cells in which these subtypes are expressed at very similar density [20]. Although receptor binding affinity of such agonists appears similar for both subtypes, it is not clear, as yet, whether the selectivity of these agents can arise as a consequence of specific conformational changes in the activated receptor leading to selective coupling, or that different receptor reserves can account for these differences. Nevertheless, there is increasing evidence that many 7TM receptors may adopt multiple active states that promote selective G-protein coupling in response to different agonists [9] and one can be certain that a careful examination of muscarinic subtype trafficking is under investigation.

Constitutive Receptor Activation and Inverse Agonists

The most widely accepted model describing G-protein-coupled receptor activation relates to the active form of the receptor as a ternary complex formed as a sequential association of ligand and G-protein in either order with the receptor [21]. However, based on many recent analyses of G-protein coupled receptor activation, this model has been revised to accommodate constitutively active states, i.e. many G-protein linked receptors exhibit spontaneous activity in the absence of agonist [22]. In this model, it is proposed that receptors exist in active and inactive forms and the binding of agonists shifts the equilibrium towards the active state that can couple with appropriate G-proteins. In contrast, antagonists may either not distinguish between active and inactive conformations and not alter the resting equilibrium (neutral antagonist) while others bind preferentially to the inactive state and shift the equilibrium to suppress constitutive activity. There is clear evidence that such constitutive activity and the action of inverse agonists are dependent on the level of receptor [23] or G-protein [24] expression. Furthermore, it has been argued that unbound receptor/G-protein complexes accumulate more readily in isolated membranes [25] and indeed inverse agonist activity of atropine can be observed using GTPγS binding to cardiac [26] or CHO m₂/m₄ membranes [27]. However, the recent demonstration of inverse agonist action in intact cells expressing relatively low densities of bradykinin B2 [28] histamine H2 [29] and m₁-m₄ muscarinic receptors [30] suggest that the phenomenon may occur quite widely in cells. Ongoing studies in several laboratories are attempting to define the conditions that determine constitutive activity of G-protein linked receptors in intact cells. Clearly, not only the cytosolic constituents such as GTP, but also the controls that are exerted at the cell membrane and/or cytoskeleton to maintain the organisation of receptors and G-proteins, are important factors in this phenomenon [12].

Finally, there is recent evidence that receptors may form multiple conformations for G-protein interactions (induced conformational pleiotropy) [31] and different active states can interact with distinct G-protein and effector systems. This again highlights receptor trafficking and the potential to design agents to selectively activate or inhibit an effector cascade by trapping or inhibiting particular conformation of the receptor. Such mechanisms, if they occur widely in cells, also have enormous implications for the classification of receptors and the development of new receptor active drugs. In particular, the possibility that most 'antagonists' are not silent could limit their apparent affinity as a 'gold standard' for receptor identification. Indeed, there

is subtype variation in the ability of various antagonists to suppress constitutive activity at m₁-m₄ recombinant receptors [30] and this could underlie the significantly different K_B values of muscarinic antagonists apparently interacting with single receptor subtypes [1].

M₃ Receptor Regulation

Recent studies from our laboratory and elsewhere have revealed that most receptors that link via Gq/11 to PIC display a very rapid, though often only partial desensitisation. The common perception therefore, that this signalling pathway is resistant to rapid homologous regulation has been dispelled, with the realisation that very early (<1 min) signalling events must be examined [32,33,34]. Thus, many G-protein linked receptors, including those that activate PIC, appear to be able to modulate their signalling and it is tempting to suggest that this could play an important rôle in the integration of information detected at a post-receptor level. Furthermore, it remains possible that cells have evolved multiple receptor subtypes that are linked to the same effector but display marked differences in their vulnerability to regulation.

Our own studies have particularly featured m₃ muscarinic receptor signalling and have recently been reviewed [32,35,36,37]. In brief, evidence that a rapid desensitisation of m₃ (and m₁) receptor signalling occurs, has come from the biphasic accumulation of Ins(1,4,5)P₃ and the non-linear accumulation of inositol phosphates in the presence of lithium when cells expressing m₃ or m₁ receptors are continuously exposed to agonist. Furthermore, the pre-exposure to agonist followed by washing resulted in a subsequent truncated response to the same agonist [33,37]. The mechanisms underlying this regulation and the possible link to our discovery of agonist-mediated phosphorylation of the m₃ and m₁ receptors is a major direction of our research at present. Here we wish to briefly describe some of our recent data on the possible rôle of precursor lipid depletion and the identification of a kinase that can phosphorylate the m₃ receptor in an agonist-dependent manner.

The second messenger Ins(1,4,5)P₃ is generated upon agonist stimulation solely from the action of a PIC on PIP₂ [3]. This key lipid substrate comprises a very minor fraction of the total membrane inositol phospholipid and it has often been emphasised that receptor mediated signalling could be compromised if the substrate becomes rate-limiting without continued or accelerated resynthesis [36]. Indeed support for the hypothesis that PIC activity can be limited by the substrate supply has come from studies with the anti-manic drug lithium. The uncompetitive inhibition of inositol monophosphatase by this monovalent ion leads to severe disruption of inositol recycling required for phosphoinositide resynthesis [38] and inability of muscarinic agonists to sustain Ins(1,4,5)P₃ accumulation in CHO cells expressing recombinant m₁ receptors [39].

Recent evidence from SH-SY5Y neuroblastoma expressing m₃ muscarinic receptors has revealed that there is a 70% depletion of PIP₂ within 1 min of receptor activation [40]. Although measurement of steady-state levels of PIP₂ do not provide indication of an increase in flux, allowing supply to match demand, it is tempting to consider that the reduced availability of PIP₂ can account for the rapid (but only partial) desensitisation frequently observed with m₃ or m₁ mediated phosphoinositide signalling [32,35,36,37]. However, it remains unclear if the effective [PIP₂] falls below its enzyme saturation upon agonist stimulation and we are still ill-informed on the compartmentalisation of such substrate pools or indeed whether more than one species of PIC is involved in a sustained signalling period. Furthermore, we have recently demonstrated that the sustained reduction in the level of PIP₂ is related to the concentration of

agonist activating m₃ receptors in SH-SY5Y cells. Thus, at sub-maximal agonist concentrations there is PIP₂ available for hydrolysis and therefore substrate supply is unlikely to underlie rapid desensitisation of the PIC response.

Very recently, we have attempted to further investigate this question of substrate supply in the desensitisation of PIC signalling by comparing responses mediated by human recombinant m₃ muscarinic receptors transfected into α T3-1 cells. This gonadotrope-derived cell line endogenously expresses a gonadotropin-releasing hormone (GnRH) receptor linked via Gq/11 to PIC. However, recent studies have revealed that this receptor does not undergo rapid (<10 min) homologous desensitisation [41]. Moreover, GnRH receptors are unique amongst the currently known 7TM receptors in that they lack the cytoplasmic C-terminal which has been established to be crucial to both receptor internalisation and desensitisation [see 37]. Our data with transfected α T3-1 cells confirm that GnRH receptor-mediated PIC activity, either assessed by Ins(1,4,5)P₃ accumulation or total inositol phosphate accumulation does not desensitise for at least 10 mins. However, crucially, agonist-stimulated m₃ receptors in the same cell display the characteristics of rapid desensitisation using both these measures of PIC activity. These data provide strong evidence that the rapid desensitisation of PIC signalling is receptor rather than cell-dependent. They also strongly imply that unless each receptor can assess its own pool of substrate, then it remains very unlikely that a rapid depletion of PIP₂ becomes rate-limiting and is responsible for the reduced PIC activity.

The above studies with the GnRH receptor have also highlighted the potential rôle of phosphorylation in agonist-mediated desensitisation. There is substantial evidence that agonist-induced phosphorylation on serine and threonine residues (often within the C-terminal) of 7TM receptors is a key feature in the uncoupling from appropriate G-proteins and probably, in some cases, in the initiation of receptor sequestration. Such phosphorylation can be mediated by protein kinase A or C or by specific G-protein coupled receptor kinases (GRKs) [see 37]. The original and classical example of such a kinase is β -ARK or (GRK-2) that phosphorylates the β -adrenoceptor at specific residues on the C-terminal allowing the binding of β -arrestin, uncoupling of the phosphorylated receptor from Gs and the reduced activation of adenylyl cyclase [42].

A large number of 7TM receptors that couple via Gq/11 to PIC have also been shown to undergo agonist-mediated phosphorylation by kinases that appear to be distinct from protein kinase C [37]. Although there is evidence that PIC linked receptors can be phosphorylated by the GRK family, studies have, in most cases, been carried out in reconstituted or transient expression systems that are entirely non-physiological. However, studies from our laboratory focusing on muscarinic receptors, have established that the m₃ receptors in CHO cells are phosphorylated on serine within seconds of agonist stimulation. The use of inhibitors have indicated that the receptor kinase is distinct from PKC, PKA and Ca²⁺/calmodulin kinase [43]. Furthermore, in a broken cell preparation the receptor kinase was, at least, partially associated with the membrane and was insensitive to zinc and heparin at concentrations that can totally suppress GRK-2 and 3 activity [44]. Recent studies [45] have identified a novel 40kDa kinase from porcine cerebellum using a strategy based on the phosphorylation of a bacterial fusion protein containing a region of the third intracellular loop of the m₃ receptor. Reconstitution of 40kDa kinase with membranes prepared from CHO-m₃ cells resulted in an increase in agonist sensitive (but not basal) phosphorylation of the intact receptor. Ongoing studies include over-expression of this kinase with m₃ receptors in various systems in order to confirm its action in agonist-sensitive phosphorylation. Furthermore, definitive evidence that m₃ muscarinic

receptor desensitisation is associated with rapid receptor phosphorylation is being investigated with mutant receptors lacking phospho-acceptor sites.

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SELECTIVE ALLOSTERIC ENHANCEMENT OF THE BINDING AND ACTIONS OF ACETYLCHOLINE AT MUSCARINIC RECEPTOR SUBTYPES

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Summary

The ternary allosteric model predicts the possibility of discovering molecules with novel and highly subtype-selective modes of action. This approach has been applied to muscarinic receptors. The alkaloid brucine is capable of selectively enhancing by an allosteric mechanism the effects of low but not high concentrations of acetylcholine at only the m1 subtype of muscarinic receptors. A simple derivative of brucine, N-chloromethylbrucine, enhances acetylcholine actions selectively at only m3 receptors. In addition it binds to, but does not affect, the properties of m4 receptors, thereby demonstrating neutral cooperativity and an 'absolute' selectivity of action at m3 receptors over m4 receptors. Brucine N-oxide enhances acetylcholine binding at m3 and m4 receptors and is neutral at m1 and m5 receptors. These findings allow the possibility of developing muscarinic agents that have a novel and highly targeted mode of action; they may act only on a single muscarinic receptor subtype which is functioning sub-optimally and therefore be of use therapeutically in the early stages of Alzheimer's Disease.

Key Words: muscarinic receptors, allosteric site, brucine, acetylcholine

Some of the cognitive deficits in the early stages of Alzheimer's Disease are thought to be associated with a localized degeneration of cholinergic nerve terminals and a consequent acetylcholine (ACh) deficit in certain brain regions(1-5). Attempts have been made to compensate for this deficit by developing anticholinesterases and muscarinic agonists as drugs (e.g. 6-9).

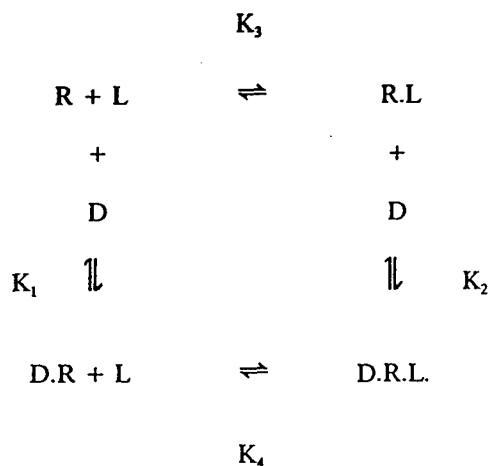
The therapeutic basis of the action of acetylcholinesterases is the prolongation of ACh actions by inhibition of its breakdown. However such prolongation will not be selective as it will take place at all acetylcholine receptors, i.e. at all the subtypes of both nicotinic and muscarinic receptors. There are as yet few, if any, examples of highly selective muscarinic agonists. A number of agonists exhibit selective "efficacy" rather than selective affinity at different muscarinic receptor subtypes (10-12). However efficacy, measured at the level of a whole cell or a whole tissue

response, is not a constant parameter which depends only on the agonist and the receptor subtype; it is highly dependent on the nature of the protein components of the signal transduction pathway, their levels, as well as the nature and position of the response along the pathway that is being measured. Both approaches therefore do not necessarily target selectively one receptor subtype and certainly not just those receptors in the damaged area of the brain.

We are exploring a third approach, that of allosteric regulation by pharmacological agents of muscarinic receptor binding and function, which may allow such selective targeting of drug action.

The ternary allosteric model

Allosteric effects are observed when there are mutual cross-interactions between two binding processes which occur simultaneously or sequentially; the binding of one ligand affects the binding of a second ligand and vice versa. The simplest scheme depicting these processes is shown below:



R represents the receptor and D and L the two ligands. Cooperativity is associated with the formation of the ternary complex, D.R.L., the magnitude of the cooperativity being defined by the ratio of the affinity constants, α , which is equal to both K_2 / K_1 and K_4 / K_3 . This scheme describes satisfactorily the allosteric interactions of gallamine and strychnine with muscarinic receptors (13,14) and has been used in the analysis of our experimental data. As we are concerned primarily with allosteric regulation of endogenous receptor function, D in the above scheme is the neurotransmitter, in this case acetylcholine (ACh), and L as the allosteric ligand.

Actions and selectivities of allosteric agents

The relative values of K_1 and K_2 define the nature of the very different possible pharmacological actions of allosteric agents. If $K_1 \gg K_2$, L behaves for all intents as a competitive antagonist and it may not be possible to determine that L is binding to a different site from D. If $K_1 > K_2$ there is low negative cooperativity between D and L and L behaves as an allosteric inhibitor whose

actions can be overcome by high concentrations of D: if $K_1 < K_2$ there is positive cooperativity and L is an allosteric enhancer which increases the binding and actions of low but not high concentrations of D. A special case occurs when $K_1 = K_2$; there is neutral cooperativity and the binding of L to R does not change the equilibrium binding of D to R.

The observed cooperativities and the resulting actions of a series of allosteric ligands therefore depend on the structure-binding relationships of the unliganded and the liganded receptors (R and D.R respectively); more precisely, it is the *differences* in the structure-binding relationships that are important. If D is the neurotransmitter, then there is a clear potential for allosteric ligands within a series to exhibit a variety of different cooperativities (positive, neutral or negative) and hence actions, if $K_1 \approx K_2$.

Allosteric ligands have the potential for being highly subtype selective agents. This selectivity may arise for two reasons. Firstly, the allosteric site may not have been conserved as stringently as the neurotransmitter binding site during evolution and therefore the values of K_1 for different receptor subtypes may be very different. Secondly, even if the allosteric site is very similar between subtypes, only a small difference in the structure of the site between the subtypes is required to change the action of a molecule from one which exhibits neutral cooperativity against a given subtype to one characteristic of positive (or negative) cooperativity at another subtype. This introduces the concept of absolute subtype selectivity, that is, a molecule which has a positive (or negative) action at one subtype and *no action* at another subtype where it has neutral cooperativity with the neurotransmitter even when the molecule is present at concentrations sufficient to saturate its binding to both subtypes.

We are particularly interested in molecules which are selective allosteric enhancers of function at one or more muscarinic receptor subtypes and which exhibit neutral or weak cooperativities at the other subtypes. Such molecules could act as lead structures for allosteric enhancers which might be of use therapeutically in the alleviation of the cognitive dysfunction resulting from the localized cholinergic deficit in the early stages of Alzheimer's Disease.

Brucine as an allosteric enhancer at m1 receptors

Previous published studies have shown that strychnine acts as an allosteric ligand at the cloned human muscarinic receptor subtypes (14,15). Furthermore we were able to demonstrate that it had a low negative cooperativity with ACh at the m1,m2 and m4 subtypes ($\alpha = 0.2 - 0.5$) (14). This indicated that a small modification of the strychnine structure might generate molecules which were positively cooperative with ACh. We have discovered that the alkaloid brucine, 10,11-dimethoxy strychnine, enhances the binding and actions of acetylcholine at human m1 receptors by an allosteric mechanism (Birdsall et al., submitted for publication).

A 1.5-2 fold positive cooperativity with ACh binding was calculated from an analysis, using the ternary allosteric model (14), of a series of [3 H]-N-methylscopolamine ([3 H]-NMS)/ACh equilibrium competition experiments performed in the presence of a series of concentrations of brucine. The affinity of brucine for the ACh-occupied m1 receptor was ca. 10^5 M^{-1} . Brucine also slowed down the dissociation rate of [3 H]-NMS from m1 receptors in a concentration-dependent manner similar to that found for strychnine (14) and other allosteric ligands (13,15-22). Although the equilibrium binding of [3 H]-NMS was little affected, the potency of brucine to affect the kinetic slowing agreed with the value calculated from its ability to enhance ACh binding in the

equilibrium competition experiment. Therefore both the equilibrium and kinetic manifestations of allosterism appear to be a consequence of the same binding events.

In assays of m1 receptor function, both in membranes ($[^{35}\text{S}]$ -GTP γ S binding) and in whole cells (elevation of cAMP and $[\text{Ca}_i]$ levels) brucine enhanced the potency of ACh 2-3 fold (Birdsall et al., submitted for publication). Brucine (100 μM) however had no significant effects on basal levels of function nor on the maximal level of stimulation by ACh and its effects on function were blocked by the muscarinic antagonist, 3-quinuclidinylbenzilate (1 μM). Brucine therefore demonstrates both qualitatively and quantitatively the same simple allosteric behavior as was found in the binding studies.

N-Chloromethylbrucine as an allosteric enhancer at m3 receptors

The positive allosteric effect of brucine on ACh binding was confined to m1 receptors. At m2-m5 receptors the values of the (negative) cooperativity varied from 0.1-0.5, depending on the subtype. However derivatives of brucine exhibited a different subtype selectivities in their allosteric actions. One quaternary analogue, N-chloromethylbrucine (CMB), enhanced the binding and membrane function ($[^{35}\text{S}]$ -GTP γ S binding) of ACh at m3 receptors 3-5 fold but not at the four other subtypes. (Birdsall et al., submitted for publication; Lazareno et al., manuscript in preparation). It was negatively cooperative with ACh at m1,m2 and m5 receptors but was essentially neutrally cooperative at m4 receptors ($\alpha = 1.1 \pm 0.1$). The neutral cooperativity at m4 receptors was found in both binding and functional studies although CMB was still clearly binding to m4 receptors as could be demonstrated by its ability to decrease the dissociation rate constant of $[^3\text{H}]\text{-NMS}$ from these receptors. N-Chloromethylbrucine therefore represents a compound which is predicted to have an element of absolute selectivity (as discussed earlier in this paper) in that it should not affect the action of ACh on m4 receptors but have actions on the other subtypes.

CMB also enhanced, in a dose-dependent manner, the submaximally field-stimulated (0.1Hz) contractions of isolated strips of guinea pig ileum (Birdsall et al., submitted for publication). The threshold concentration of CMB for observing the potentiation was 2-6 μM and up to a 300% enhancement could be observed at higher concentrations. This whole tissue response was totally blocked by atropine (300nM) but was unaffected by hexamethonium (40 μM) and, although eserine (4-400nM) produced a comparable enhancement of the contractions, CMB at the concentrations used (up to 300 μM) had no anticholinesterase activity. It therefore seemed that CMB was potentiating the actions of ACh in a whole tissue response mediated via muscarinic receptors. However the potentiation could have been due to CMB acting in an allosteric inhibitor at presynaptic inhibitory M_2 receptors rather than as an allosteric enhancer at postsynaptic M_3 receptors. The former possibility was eliminated by the fact that the M_2 / m2 selective antagonist methoctramine did not enhance the field stimulated contractions (under these conditions) at any of the concentrations examined (1nM-5 μM) although it did inhibit contractions at the higher concentrations when it is known to inhibit M_3 /m3 receptors. The evidence is therefore that CMB can act as an allosteric enhancer at postsynaptic M_3 receptors.

Brucine N-oxide as an allosteric enhancer at m3 and m4 receptors

Brucine N-oxide has been shown to exhibit a different subtype selectivity from CMB and brucine. It is positively cooperative with ACh at both m3 and m4 receptors, essentially neutral at m1 and m5 receptors and weakly negatively cooperative at m2 receptors (Birdsall et al., submitted for

publication). This pattern of cooperativity is seen in both binding and functional studies in membranes. Other brucine derivatives are also capable of acting as allosteric enhancers at muscarinic receptor subtypes (23).

Allosterism in soluble receptor preparations

Preliminary studies have shown that the allosteric enhancement of ACh binding by brucine N-oxide at m4 receptors and CMB at m3 receptors is retained in digitonin solubilised preparations and the enhancement by brucine at m1 receptors is observed in receptors which had been substantially purified by sequential DEAE-Sepharose, affinity and hydroxyapatite chromatography using the methodology described by Haga, Haga and Hulme (24). This suggests that the allosteric site for these ligands is located on the receptor protein itself and not on another protein or the lipid membrane. This conclusion is reinforced by the results of Curtis and Hulme (25) who have demonstrated that the purification of the [3 H]-NMS complex of an epitope tagged m1 receptor expressed in *E. Coli* is facilitated by stabilization of the [3 H]-NMS-bound receptor by strychnine.

Conclusions

Brucine and its analogues bind to muscarinic receptors and regulate ACh actions according to the ternary allosteric model. An enhancement of ACh binding and function has been demonstrated at m1, m3 and m4 receptors. The positive cooperativities are relatively small but nevertheless are of a magnitude comparable to those found for benzodiazepines acting on GABA_A receptors (26-29). Small changes in the structure of the brucine analogues change the subtype specificity of their allosteric actions such that a variety of molecules can be generated which have a spectrum of positive, negative and neutral cooperativities at the different receptor subtypes. Neutral cooperativity at a specific subtype is a beneficial characteristic of an allosteric agent if it is desired that the agent have no actions at that subtype. Although the brucine analogues bind with relatively low affinity to muscarinic receptors, their demonstrated selectivities and abilities to enhance the actions of low concentrations of ACh suggest that a more potent allosteric muscarinic enhancer with an appropriate subtype selectivity might be useful in the treatment of Alzheimer's Disease.

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DISCOVERY & DEVELOPMENT OF SELECTIVE M₃ ANTAGONISTS FOR CLINICAL USE

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Summary

The treatment of airway obstructive disease may be improved by antimuscarinic agents which selectively block M₁ and M₃ receptors but do not inhibit prejunctional cholinergic autoreceptors which limit release of acetylcholine. Revatropate is a novel antimuscarinic agent which shows some 50-fold selectivity for M₁ and M₃ receptors in guinea pig trachea and rabbit vas deferens over the M₂ subtype in atria. This selectivity profile was seen *in vivo* in anaesthetised guinea pigs and conscious dogs where bronchodilator activity was produced in the absence of any effect on heart rate. Revatropate, in contrast to the non-selective agent ipratropium, did not potentiate bronchoconstrictor responses induced by vagal nerve stimulation, indicating that inhibitory autoreceptors were still functional. Early clinical studies in COAD patients showed that inhaled revatropate was an effective bronchodilator which was well tolerated. Darifenacin differs from revatropate by showing selectivity for M₃ receptors relative to both M₂ and M₁ subtypes. [³H] darifenacin had 5-fold higher affinity for the human m₃ relative to m₁ receptors while there was significantly reduced binding to m₂, m₄ and m₅ receptors. The degree of selectivity in functional tissue preparations was even greater, with darifenacin showing 100-fold selectivity for the ileum M₃ receptors over M₂ receptors in atria and 30-fold over M₁ receptors in rabbit vas deferens. Darifenacin was able to differentiate between M₃ receptors in different tissues; although darifenacin was equipotent with atropine in the ileum and bladder, it was some 10-fold and 6-fold less potent at inhibiting muscarinic responses in the trachea and submandibular salivary gland respectively, relative to atropine. Studies in anaesthetised dogs confirmed this selectivity profile. Thus darifenacin inhibited responses of the gut and bladder to cholinergic stimulation without affecting heart rate. Salivary gland responses were inhibited at doses some 6-10 fold higher than those required to inhibit gut and bladder responses. Clinical studies are ongoing in urge incontinence and functional bowel disease which may confirm this selectivity profile.

Key Words: muscarinic receptor subtypes, M₃ antagonists, preclinical pharmacology

The *Datura* genus of plants which contain atropine and related anticholinergic alkaloids, have been used by man for several thousand years for a variety of medical and other purposes. Thus for example, inhalation of smoke from the herb *Datura stramonium* was recommended for the treatment of asthma in the seventeenth century (1). However, drying of secretions, tachycardia, urinary retention, blurred vision and central nervous system effects were all recognised complications associated with the use of atropine containing compounds and preparations. Muscarinic antagonists are still widely used, for example oxybutynin in urinary urge incontinence and dicyclomine in irritable bowel syndrome, but the clinical utility of these non-selective agents is still limited by adverse side effects.

In 1976, Barlow's studies of the relative activities of a series of muscarinic antagonists in the guinea pig ileum and the rabbit atrium indicated that responses in these two tissues were mediated by different receptors (2). Further work clearly established the heterogeneity of muscarinic receptors and they have been classified using pharmacological techniques into M₁, M₂, M₃ and M₄ subtypes, and on genomic cloning into m1, m2, m3, m4 and m5 subtypes (3). Thus the possibility of designing sub-type selective agents to achieve efficacy with reduced side-effects was a feasible and exciting possibility. Muscarinic M₃ receptors are located predominantly on smooth muscle and salivary glands, and it was considered that agents selective for this sub-class of receptors could have therapeutic utility in the treatment of incontinence, disorders of gastro-intestinal motility and as bronchodilators in respiratory disease. We therefore initiated projects at Pfizer to identify M₃ selective antagonists and this paper describes the profile of 2 such agents, revatropate (UK-112,166) and darifenacin (UK-88,525) (Fig. 1).

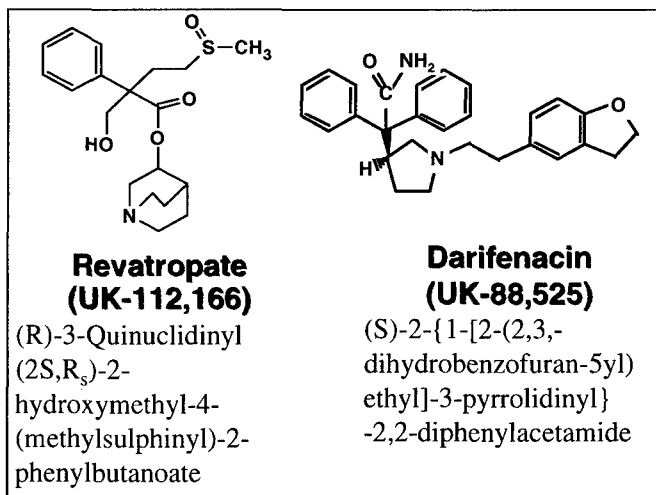


Fig. 1
Structural formulae of novel M₃ antagonists.

Revatropate: - a drug for treatment of COAD

Activation of cholinergic nerves is the major bronchoconstrictor neural mechanism in animal and human airways and there is evidence that this mechanism may be overactive in

muscarinic receptors M₁, M₂, and M₃ have been identified in human airways and their location and possible physiological function have been extensively reviewed (5). Stimulation of the vagus nerve releases acetylcholine (ACh) which activates muscarinic receptors on airway smooth muscle (M₃) and submucosal gland (M₃) to induce bronchoconstriction and mucus secretion respectively. M₁-receptors facilitate neurotransmission through parasympathetic ganglia and possibly enhance cholinergic reflexes. Additionally, there are also autoreceptors on cholinergic nerve terminals innervating bronchial and tracheal smooth muscle which inhibit ACh output when activated (6, 7) which have been characterised as the M₂ muscarinic subtype (5, 8). (In the guinea pig, there is some evidence that these autoreceptors may be more M₄-like rather than M₂ (9)). Thus a rational approach to producing an improved agent to treat airways obstruction was to identify a compound which selectively inhibited M₃ and M₁ muscarinic receptors, but unlike non-selective agents such as ipratropium, did not interfere with the neurotransmitter negative feedback loop by blockade of M₂ (or M₄)-receptors.

The *in vitro* profile of revatropate is shown in Table I. Revatropate showed potent antagonism of M₁ and M₃ receptors with some 50-fold selectivity over the M₂ subtype. This was in contrast to ipratropium which was also potent, but non-selective (10).

TABLE I

In vitro profile of revatropate and ipratropium.

TISSUE/SUBTYPE	REVATROPATE			IPRATROPIUM		
	pA ₂ (95% conf. limits)	slope	n	pA ₂ (95% conf. limits)	slope	n
Rabbit vas deferens M ₁	9.26 (8.93-9.93)	1.06	11	9.44 (9.16-9.86)	1.11	14
Guinea pig atria M ₂	7.25 (7.12-7.39)	1.19	16	9.15 (8.91-9.45)	1.21	4
Guinea pig trachea M ₃	8.92 (8.67-9.24)	0.97	17	9.45 (9.24-9.73)	1.38	12

n=number of tissues

This selectivity profile was also seen *in vivo*. Thus in anaesthetised guinea pigs, revatropate antagonised ACh-induced M₃ mediated bronchoconstrictor responses at some 80-fold lower doses than those required to inhibit ACh-induced M₂ mediated bradycardia (10). Ipratropium inhibited both responses over a similar dose range.

The functional involvement of pre-junctional muscarinic receptors on post-ganglionic airway cholinergic nerves that inhibit the release of ACh in lung responses to nerve stimulation has been demonstrated in animal experiments (5, 8, 11). For example, bronchoconstrictor responses to vagal nerve stimulation were potentiated by the M₂/M₄-selective antagonist methoctramine, indicating inhibition of the inhibitory feedback control on ACh release. Similar experiments were carried out in our laboratories in anaesthetised guinea pigs and cats with revatropate and ipratropium, comparing their effect on bronchoconstriction induced by vagal stimulation with responses to injected ACh (12). Ipratropium potently inhibited i.v. ACh-induced bronchoconstriction in both species (ID₅₀ 1.45 µg/kg in guinea pig, 0.08 µg/kg in cat). Significantly higher doses were required to inhibit responses to vagal nerve

stimulation ($ID_{50} > 10 \mu\text{g/kg}$ in guinea pig, $2.1 \mu\text{g/kg}$ in cat). Additionally, low doses of ipratropium potentiated the bronchoconstriction responses to vagal-nerve stimulation by up to 150%. (See Fig 2). Revatropate also potently inhibited the bronchoconstrictor response to i.v. ACh (ID_{50} $0.94 \mu\text{g/kg}$ in guinea pig and $0.52 \mu\text{g/kg}$ in cat). However, in contrast to ipratropium, responses to vagal stimulation were similarly inhibited over the same dose range (ID_{50} $1.7 \mu\text{g/kg}$ in guinea pig, $1.03 \mu\text{g/kg}$ in cat). Potentiation of the vagal response was not observed at any dose. The data from guinea pigs are shown in Fig 2.

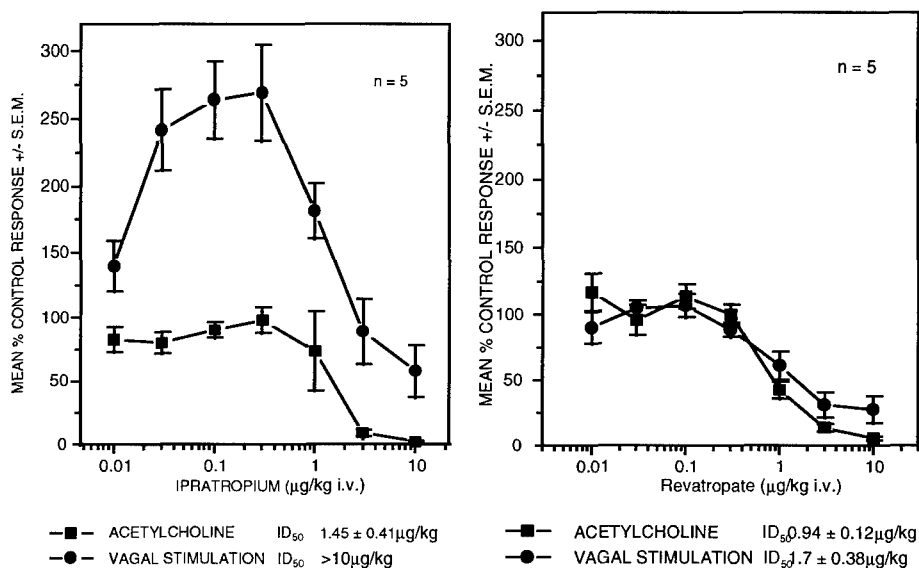


Fig. 2

Selectivity of revatropate for post-junctional M_3 receptors over pre-junctional autoreceptors - comparison with ipratropium in anaesthetised guinea pigs.

The bronchodilator activity and lung selectivity of revatropate was confirmed in conscious dogs where revatropate, given orally and by metered-dose inhaler, antagonised nebulised methacholine-induced bronchospasm without affecting heart rate (13).

The role of the autoreceptor on pre-junctional cholinergic nerve terminals in the airways in man is unknown, but indirect clinical pharmacology studies indicate their presence in non-asthmatics but absence in asthmatic subjects (14). It is possible, that while neuronal M_2 receptors exert an important inhibitory control on the parasympathetic nerves supplying the airway smooth muscle in normal circumstances, the situation may be different in the disease state. Thus measurements of vagal-induced bronchoconstriction responses in guinea pig indicated that prejunctional autoreceptors are dysfunctional after exposure to viral airway infections, ozone or antigen inhalation (15). Inflammatory mediators and/or eosinophil degranulation products were implicated in the loss of M_2 function in these studies.

The first clinical study evaluating the effect of revatropate in COAD patients was very encouraging. Forty two COAD patients were studied in a double-blind 3 treatment cross-over study, designed to compare the response to $320 \mu\text{g}$ revatropate, $80 \mu\text{g}$ ipratropium bromide and

placebo given via Meter Dose Inhaler (16). The mean increase from pre-dose FEV₁ to peak-post dose FEV, was 0.34L (+27%) on revatropate, 0.36L (+29%) on ipratropium bromide and 0.15L (+12%) on placebo. Revatropate was well tolerated and no side effects were reported. Further studies are required to explore the dose-response relationships, and these may reveal advantages over non-selective agents.

Darifenacin

The functional smooth muscle responses induced by cholinergic nerve stimulation to the bladder and gut are mediated via M₃ receptor activation (3). Darifenacin differs from revatropate by showing selectivity for the M₃ subtype relative to all other muscarinic receptor subtypes and hence is anticipated to have an advantage over non-selective anti-muscarinic agents in the treatment of urge incontinence and functional bowel disease.

The binding of [³H]-darifenacin to the five cloned human muscarinic receptors (m1 - m5) expressed in CHO cells was compared (17). [³H]-darifenacin was found to bind with 5-fold higher affinity to m3 (K_D, 0.33nM) over m1 (K_D, 1.6nM) receptors. There was no specific binding to m2 receptors and specific binding to m4 and m5 receptors was insufficient to determine a K_D. Competition studies in cells expressing m3 and m1 receptors using a range of muscarinic antagonists showed that darifenacin represents the first selective m3 ligand. A greater degree of selectivity for the M₃ receptor subtype was observed in functional *in vitro* studies. The pA₂ values for darifenacin and atropine versus muscarinic activation in a variety of tissue preparations are shown in Table II. Atropine was essentially non-selective while darifenacin showed 100-fold selectivity for ileum M₃ receptors over M₂ receptors in atria and 30-fold over M₁ receptors in rabbit vas deferens (18, 19).

TABLE II

In vitro profile of darifenacin and atropine.

TISSUE/SUBTYPE	DARIFENACIN			ATROPINE		
	pA ₂ ± sem	slope	n	pA ₂ ± sem	slope	n
Guinea pig ileum M ₃	9.44 ± 0.07	1.16	6	9.4 ± 0.07	0.96	6
Guinea pig trachea M ₃	8.70 ± 0.09	1.08	6	9.20 ± 0.1	1.18	6
Guinea pig bladder M ₃	8.66 ± 0.14	1.01	4	9.01 ± 0.09	0.86	4
Guinea pig atria M ₂	7.48 ± 0.13	0.84	6	8.72 ± 0.06	1.13	7
Rabbit vas deferens M ₁	7.90 ± 0.08	0.94	6	9.58 ± 0.09	0.9	7
Guinea pig salivary gland M ₃	7.0 ± 0.1 (pIC ₅₀)	-	4	7.87 ± 0.14 (pIC ₅₀)	-	4

Functional responses to cholinergic activation of salivary glands are also mediated by the M₃ muscarinic subtype. However, although darifenacin was equipotent with atropine in the ileum and bladder preparations, darifenacin was some 6-fold less potent at inhibiting carbachol-induced ⁸⁶Rb efflux from the submandibular salivary gland and 10-fold less potent at

inhibiting ACh induced contractions in trachea, relative to atropine. Thus, when compared with atropine, darifenacin showed a degree of selectivity for the ileum relative to other smooth muscle preparations studied and also relative to the salivary gland responses.

Other muscarinic antagonists have also been reported to differentiate between M₃ receptors expressed in different tissues, for example hexahydrosiladifenidol (20) and zamifenacin (21). The molecular basis for this selectivity is not understood, especially since only one M₃ receptor has been identified from molecular sequence studies. However, the pharmacological data suggests that there may be some heterogeneity of conformation when expressed in the native tissues. It is possible that receptor configuration or antagonist binding are affected by tissue dependent factors such as signal transduction mechanisms, or the presence of other receptor subtypes in the tissue membrane. Thus, although it is well established that the functional receptor mediating contraction in the trachea, ileum and bladder is of the M₃ subtype, the major population of muscarinic receptors in the smooth muscle of these tissues is of the M₂ subtype (3). The role of M₂ receptors in these smooth muscle preparations is unknown, but it is conceivable that their presence and primed state (linked to adenylylase) may modulate post receptor events in a tissue-dependent way. The availability of tritiated darifenacin should help to identify any differences in tissue binding and could provide useful data for receptor modelling and mutagenesis studies.

Studies in anaesthetised dogs confirmed the potent M₃ antagonist activity of darifenacin on gut and bladder and the clear selectivity over M₂ cardiac muscarinic receptors (22). Thus darifenacin potently inhibited cholecystokinin (CCK-8) induced jejunal motility (ED₅₀ 7.5 µg kg⁻¹) whilst it was significantly weaker at inhibiting nerve induced salivary flow (ED₅₀ 54 µg kg⁻¹). Importantly, doses up to 250 µg kg⁻¹ had no effect on heart rate. In contrast, atropine inhibited CCK-8 induced jejunal motility and salivary flow over the same dose range and these doses induced marked tachycardia. The data are summarised in Fig 3.

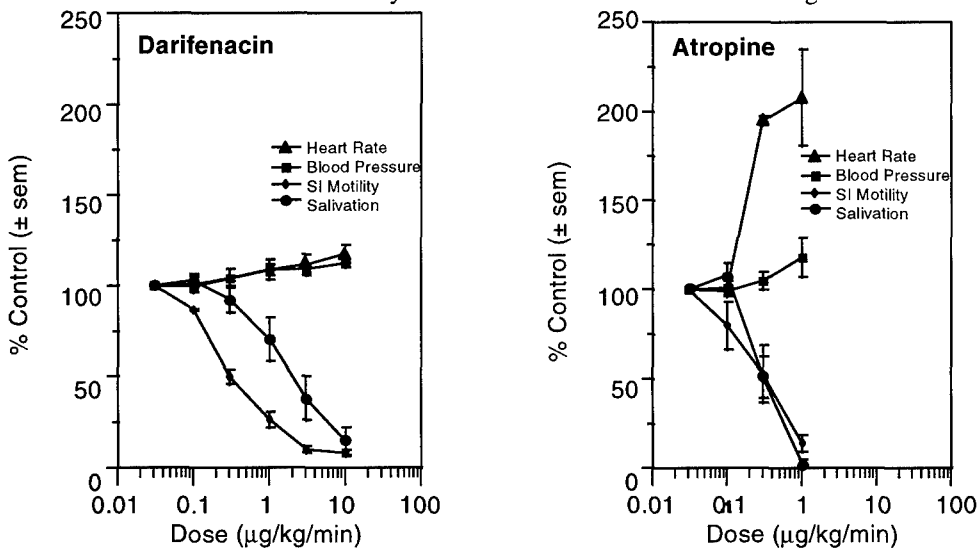


Fig.3

Effect of darifenacin and atropine on jejunal motility, salivation, heart rate and blood pressure in anaesthetised dogs.

Similar studies were carried out in anaesthetised dogs measuring contractions of the bladder to pelvic nerve stimulation (23). Darifenacin showed a 9-fold selectivity for bladder responses (ED_{50} $6.8\mu\text{g kg}^{-1}$) over salivary gland responses (ED_{50} $58.3\mu\text{g kg}^{-1}$) and had no effect on heart rate at doses up to $100\mu\text{g kg}^{-1}$. Again this was in contrast to atropine which was non-selective over the dose range $1\text{--}40\mu\text{g kg}^{-1}$. Thus the *in vitro* profile of darifenacin and its clear selectivity for gut and bladder relative to effect on to salivary gland, translated to the *in vivo* situation.

Darifenacin is currently in Phase II evaluation in urinary incontinence and functional bowel disease and early results are encouraging. A pilot study in patients with detrusor instability, assessed by continuous ambulatory monitoring, has shown that multiple doses of darifenacin were well tolerated and reduced total number, maximum amplitude, and duration of unstable bladder contractions (24).

Ongoing and future research should help to rationalise these pharmacological findings and give a better understanding of how subtypes of muscarinic receptors function in different organs both physiologically and pathophysiologically. It may then be possible to design muscarinic antagonists with even greater receptor and tissue selectivity, providing therapeutic agents with further improvements in efficacy and toleration.

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MUSCARINIC CONTROL OF AIRWAY FUNCTION

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Summary

Muscarinic M_1 , M_2 , and M_3 receptor subtypes have been shown to be involved in the pre- and postjunctional control of airway diameter of various species, including man. In a guinea pig model of allergic asthma, the prejunctional M_2 receptor was shown to become dysfunctional already during the early allergic reaction, thereby contributing to exaggerated vagal reflex activity and airway hyperreactivity. Moreover, a deficiency of endogenous nitric oxide was observed after allergen provocation, which may also contribute to an enhanced postjunctional M_3 receptor-mediated cholinergic response. Both in human and in animal airway preparations it was shown that enhanced cholinergic contractions are relatively resistant to β -adrenoceptor-mediated relaxation. The reduced β -adrenoceptor function may primarily be due to transductional cross-talk between PI metabolism and adenylyl cyclase, including protein kinase C-induced uncoupling of the β -adrenoceptor from the effector system. Cross-talk between postjunctional M_2 receptor-mediated inhibition and β -adrenoceptor-induced activation of adenylyl cyclase appears to be of minor functional importance, but could be enhanced in allergic asthma due to increased expression of the inhibitory G protein as induced by cytokines.

Key Words: M_1 muscarinic receptors, M_2 muscarinic receptors, M_3 muscarinic receptors, prejunctional muscarinic M_2 receptor dysfunction, β -adrenoceptor, receptor cross-talk, airway function

Muscarinic receptor subtypes have been shown to be involved in the control of airway smooth muscle tone and airway diameter in both animals and man. Thus, M_1 receptors are localized in parasympathetic ganglia (summarized in ref. 1) where they facilitate neurotransmission, and to alveolar walls (reviewed in ref. 2), where their function remains unclear. M_2 (-like) receptors are found prejunctionally on parasympathetic cholinergic nerve endings (3-5), where they serve to control neurotransmitter release, and M_2 as well as M_3 receptors are found in smooth muscle (2). M_4 receptors may be localized to small airways smooth muscle and alveolar walls in rabbit lung, but have not been detected in other species (2). Among the smooth muscle muscarinic receptor subtypes, the minor population of M_3 receptors play a major role in producing contraction, as has been shown in animal and human isolated airway smooth muscle preparations as well as in mouse and guinea pig lung *in vivo* (2).

Muscarinic receptor dysfunction and obstructive airway diseases

The involvement of muscarinic receptor subtypes in the control of airway diameter has prompted investigations as to whether alterations in the function of these receptors may contribute to increased airway constriction and airway reactivity in obstructive airway diseases. Thus, an increased contribution

of M₁ receptors to ganglionic transmission in cholinergic pathways may be envisaged. Such increased role was not found, however, in guinea pigs after sensitization to and challenge with ovalbumin, even though there was some airway hyperreactivity as observed with vagally induced airway constriction (6).

In contrast to ganglionic M₁ receptors, there have been a number of reports suggesting that inhibitory prejunctional muscarinic autoreceptors, may play a role in increased airway reactivity. These receptors were originally identified in guinea pig airways *in vivo* (3), and have subsequently been demonstrated, employing both vagal nerve stimulation (VNS) and electrical field stimulation (EFS) induced airway constriction *in vivo* or *in vitro* as well as direct measurement of the release of acetylcholine, in all species investigated, including man (7-9). From these studies it appears that prejunctional muscarinic autoreceptors function in extra- as well as in intrapulmonary airways (9). The subtype of muscarinic autoreceptor on cholinergic nerve terminals in the airways was initially taken to be M₂, based on the observation that M₂-selective muscarinic antagonists such as gallamine and methoctramine increased VNS- or EFS-induced constriction (3,8). Moreover, a clear and significant correlation was observed between the potencies of four M₂ selective compounds to increase EFS-induced cholinergic twitch contractions in guinea pig trachea and their apparent affinities at cardiac M₂ receptors (5). However, from the measurement of pre-labeled [³H]-acetylcholine release the involvement of M₄ rather than M₂ receptors was inferred (4,10). Recent studies appear to indicate that either M₂ and M₄ receptors are involved jointly, or that the receptors are of the M₂ type with a pharmacological profile distinct from cardiac M₂ receptors (see abstract # 66). The subtype of the prejunctional muscarinic autoreceptor in human airways has been taken as M₂ (7,9), although the (additional) involvement of M₄ receptors has not yet been analyzed.

Prejunctional muscarinic inhibitory autoreceptors in the airways appear dysfunctional in mild human asthma (11). Such dysfunction would contribute to airway constriction and airway reactivity since the braking mechanism on acetylcholine release is lost. Dysfunctional muscarinic autoreceptors have also been observed in animal models of inflammatory airway disease. Thus, exposure to antigen, virus or ozone, which all result in some type of airway inflammation and in increased airway reactivity, has been shown to result in a marked inhibition of gallamine-mediated increases and pilocarpine-mediated decreases of VNS-induced airway constriction in guinea pigs *in vivo* (12). Similarly, antigen exposure resulted in increased EFS-induced tracheal reactivity and acetylcholine release in mice, and gallamine and pilocarpine modulation of release were completely lost (13).

In our laboratory, sensitization to and single low-dose challenge with ovalbumin has been shown to induce guinea pigs to develop early (0-5h after challenge) and late phase (8-23h after challenge) bronchoconstrictive responses as well as bronchial hyperreactivity to histamine and methacholine, and the influx of inflammatory cells, more specifically eosinophils and neutrophils, into the lung (14); these characteristics are clearly reminiscent of human allergic asthma. Prejunctional muscarinic autoreceptor function in these animals was almost completely lost already after the early allergic reaction, both when measuring EFS-induced tracheal contraction *ex vivo* (15) and VNS-induced airway constriction *in vivo* (16), but was almost completely restored after the late allergic response. The significant correlation between the magnitude of the early allergic response and the severity of the prejunctional receptor dysfunction in a given animal may be taken to suggest that these phenomena result from a common origin, i.e. the influx and activation of inflammatory cells in the lung (see further below). The functional consequences of the muscarinic autoreceptor dysfunction may be inferred from the observation that bronchial hyperreactivity to histamine showed a similar time course (3.1-fold after the early, 1.6-fold after the late reaction). By analyzing the effect of ipratropium bromide on histamine-induced airway responses it was indeed established that the vagal reflex component of the histamine response was exaggerated after the early allergic reaction, indicating that prejunctional receptor dysfunction substantially contributes to bronchial hyperreactivity to histamine at this time point (17).

As to the mechanism of prejunctional receptor dysfunction after antigen challenge, eosinophil-derived major basic protein and other polycationic proteins like eosinophil peroxidase and eosinophil cationic protein have been implicated. Thus, it has been shown that prejunctional receptor dysfunction can be restored by the administration of macromolecular poly-anionic substances like heparin and poly-L-glutamate to antigen-challenged guinea pigs, which putatively remove the cationic proteins by complexation, and that major basic protein is an (allosteric) antagonist of muscarinic M_2 but not M_3 receptors (12). Concerning the putative involvement of eosinophil-derived products in prejunctional muscarinic autoreceptor dysfunction after the early allergic response in our antigen-challenged guinea pigs, increased numbers of eosinophils and increased levels of eosinophil peroxidase activity were detected in bronchoalveolar lavage already at this early time point (18), indicating that activated eosinophils are indeed present during the early response.

Another important mechanism whereby polycationic proteins may cause bronchial hyperreactivity is damage of the airway epithelium (19). This could contribute to the observed increase in vagal reflex activity in allergen-challenged guinea pigs by disrupting the diffusion barrier for inhaled stimuli to the sensory nerve endings. In addition, epithelial damage could interfere with the production of epithelium-derived relaxing factors, including nitric oxide (NO), thereby causing an exaggerated response to contractile stimuli, including cholinergic agonists. In perfused intact tracheal preparations from unchallenged ovalbumin-sensitized guinea pigs, we have recently demonstrated that the NO synthase inhibitor N^G -nitro-L-arginine methyl ester (L-NAME) markedly enhanced the maximal airway constriction induced by intra- or extraluminally applied methacholine and histamine, indicating that (agonist-induced) NO production is counteracting the contractile responses (20). Very remarkably, similar increases in methacholine and histamine responsiveness were found in tracheal preparations of guinea pigs obtained at 6 h after allergen challenge. Since L-NAME was without effect on these responses, it was concluded that a deficiency of endogenous NO contributes to allergen-induced airway hyperreactivity to cholinergic and non-cholinergic stimuli after the early asthmatic reaction. However, histological examination of the intact tracheal preparations indicated that the deficiency of NO was not due to epithelial shedding, despite the presence of epithelial and subepithelial infiltration of eosinophils (20).

In contrast to the perfused tracheal preparations, many studies using human or animal isolated airway ring or strip preparations (reviewed in 21) have indicated that (allergen-induced) airway hyperreactivity to cholinergic and other contractile stimuli *in vivo* is not due to enhanced (muscarinic M_3) receptor-mediated contractility of the airway smooth muscle itself. However, both human and animal studies have indicated that exaggerated cholinergic stimulation of airway smooth muscle in asthma may lead to a reduced relaxability of the muscle by β -adrenoceptor agonists, which may involve transductional cross-talk between postjunctional M_3 (and possibly M_2) muscarinic receptors and β -adrenoceptors, as discussed below.

Muscarinic receptor signal transduction in airway smooth muscle

Both in animal (22-24) and in human (25) airway smooth muscle it has been demonstrated that phosphoinositide (PI) metabolism induced by various contractile stimuli, including muscarinic agonists, may be involved in the pharmacomechanical coupling of contraction. In bovine tracheal smooth muscle (BTSM), this was indicated by a direct relationship between the efficacy of different muscarinic agonists to induce inositol phosphate (IP) accumulation and their potency to induce contraction, with a remarkable reserve of IP production by the full agonists methacholine and oxotremorine, and no reserve for the partial agonist McN-A-343 (23). In the same tissue, we have demonstrated that muscarinic agonist-induced PI metabolism is specifically mediated by M_3 -muscarinic receptors (24), which is in accordance with the contractile studies.

Stimulation of muscarinic M_3 receptors leads to activation of phosphoinositide-specific phospholipase C, which hydrolyses phosphoinositol 4,5-bisphosphate to generate inositol 1,4,5-trisphosphate (IP_3) and *sn*-1,2-diacylglycerol (DAG). IP_3 mobilizes Ca^{2+} from intracellular stores, which is involved in a rapid and transient rise in intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) and initiation of contraction. In addition, a sustained influx of extracellular Ca^{2+} through receptor-operated Ca^{2+} channels and DAG-induced activation of protein kinase C (PKC) have been implicated in the tonic phase of contraction (26,27). However, using the specific PKC inhibitor GF 109203X, we have recently demonstrated that activation of PKC exerts a feedforward control of methacholine- as well as histamine-induced Ca^{2+} mobilization and influx, suggesting that PKC may be involved in *both* phasic and tonic contraction induced by these agonists (28). This was underscored by a reduced initial rate of both methacholine- and histamine-induced BTSM contractions in the presence of GF 109203X, while the pD_2 of methacholine-induced contraction and both pD_2 and E_{max} values of histamine-induced contraction were also reduced (unpublished). The differential effect of GF 109203X on methacholine- and histamine-induced maximal contractions may well be explained by the lower IP accumulation and subsequent Ca^{2+} -response induced by histamine (29).

It has been established that the M_2 -muscarinic receptors are coupled to inhibition of adenylyl cyclase, presumably via the inhibitory G protein, G_i (30,31). As an additional biochemical response, G_i -mediated inhibition of large conductance Ca^{2+} -dependent potassium (K_{Ca}) channels has been put forward (32). Although muscarinic M_2 receptor stimulation is probably not important in the contraction of most airway smooth muscle tissues (2), it has been implicated in functional antagonism between cholinergic agonist-induced contraction and β -adrenoceptor-mediated relaxation, which involves stimulatory G-protein (G_s)-mediated activation of adenylyl cyclase as well as cAMP-dependent and cAMP-independent opening of K_{Ca} channels (see below).

Cross-talk between muscarinic receptor stimulation and β -adrenoceptor function in airway smooth muscle

It has been established that the potency and degree of relaxation induced by β -adrenoceptor agonists are gradually reduced in the presence of increasing concentrations of contractile agonists (reviewed in 21). Several studies have indicated that the β -adrenergic responsiveness is not only determined by the contraction level *per se*, but also by the contractile agonist under investigation, especially at higher and supramaximal concentrations. Thus, in guinea pig tracheal preparations we have found that the decrease of isoprenaline-induced relaxation in terms of pD_2 and E_{max} at increasing concentrations of the contractile stimulus was relatively large for methacholine, intermediate for oxotremorine and histamine, and only small for the partial muscarinic agonist McN-A-343 (33).

Role of PI metabolism in the functional antagonism of airway smooth muscle contraction by β -adrenoceptor agonists. Since the agonists mentioned above all have a different capacity to induce IP accumulation in BTSM slices (23,33), we considered the possibility that (the reserve of) PI metabolism by these agonists is involved in the functional antagonism by β -adrenoceptor agonists. Accordingly, we found that the efficacy of the contractile agonists to induce IP production (methacholine > oxotremorine > histamine > McN-A-343) showed a striking correlation with their capacity to reduce pD_2 and E_{max} values of isoprenaline-induced relaxation of guinea pig trachea (33). Similarly, in bovine tracheal (unpublished) and human bronchial (25) smooth muscle significant correlations between IP production induced by various concentrations of methacholine and histamine and the reduction of isoprenaline pD_2 and E_{max} values were found. These findings clearly suggest a functional relationship between the levels of PI metabolism and of β -adrenoceptor function in both animal and human airway smooth muscle. Two possible

explanations could account for this relationship: (i) competition of the two transduction mechanisms for the functional response at a post-transductional level, and (ii) direct interference of the PI metabolism with the β -adrenoceptor-mediated activation of adenylyl cyclase and *vice versa*.

In a number of tissues, it has been demonstrated that activation of PKC via PI metabolism or phorbol esters may lead to desensitization of β -adrenoceptor-mediated adenylyl cyclase activity, presumably via phosphorylation of the β -adrenoceptor and/or the stimulatory G-protein (G_s) (34). Direct evidence for a role of PKC in the functional antagonism of both muscarinic agonist- and histamine-induced contraction by β -adrenoceptor agonists in airway smooth muscle was obtained by our recent observation that isoprenaline-induced relaxation of BTSM contraction is significantly potentiated in the presence of the specific PKC inhibitor GF 109203X. In contrast to isoprenaline, forskolin- and 8-Br-cAMP-induced responses were virtually unaffected (Figure 1), indicating that uncoupling of the β -adrenoceptor from the effector system by PKC may be involved.

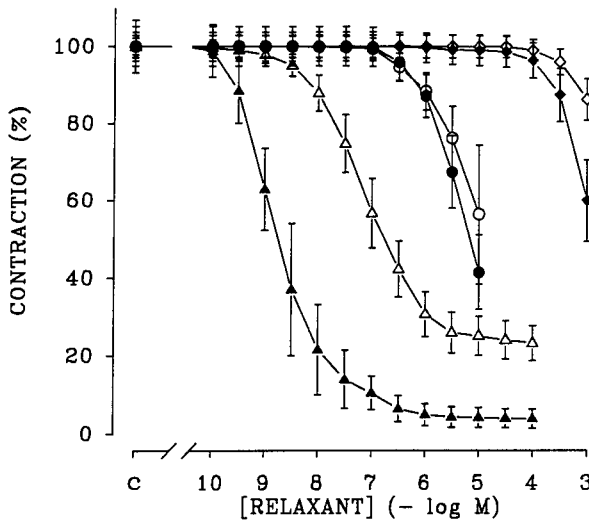


Fig. 1.

Normalized isoprenaline- (triangles), forskolin- (circles), and 8-Br-cAMP- (diamonds) induced relaxations of BTSM contraction induced by 1 μ M methacholine, in the absence (open symbols) and presence (closed symbols) of 10 μ M GF 109203X. Means \pm SEM of 4 experiments.

Modulation of agonist-induced PI metabolism and Ca^{2+} signalling by cAMP dependent mechanisms. Another mechanism of transductional cross-talk that might be involved is cAMP-dependent inhibition of PI metabolism. Some studies, using canine or bovine tracheal smooth muscle slices, have indicated that β -adrenoceptor agonists, as well as forskolin and 8-Br-cAMP, inhibit histamine-, but not muscarinic agonist-induced IP accumulation (35), which suggested that the relative resistance of muscarinic airway smooth muscle contraction to relaxation by β -agonists would be due to a lack of effect of these agonists on muscarinic agonist-induced PI metabolism.

In isolated BTSM cells, we similarly found that methacholine-induced IP accumulation was not inhibited by preincubation with isoprenaline, forskolin and 8-Br-cAMP, while histamine (up to 100 μ M)-induced PI metabolism was inhibited to only a small degree by forskolin and 8-Br-cAMP but not by isoprenaline (29). By contrast, in the same study we found marked inhibitory effects of

the cAMP-elevating agents on both methacholine- and histamine-induced Ca^{2+} mobilization and influx. As observed with contraction, these effects were strongly dependent on the concentration and nature of the contractile agonist used, with the high concentration of 100 μM methacholine being resistant to cAMP-mediated inhibition as compared to 1 μM methacholine and 100 μM histamine. Accordingly, we found a striking correlation between the isoprenaline-induced inhibition of BTSM contraction and inhibition of Ca^{2+} mobilization or influx, indicating that attenuation of $[\text{Ca}^{2+}]_i$, but not of PI metabolism, plays a major role in β -adrenoceptor-mediated relaxation of methacholine- and histamine-induced contraction. As with PI metabolism, a large reserve of Ca^{2+} mobilization and influx for methacholine-induced contraction at the supramaximal concentration of 100 μM was found, indicating that the relative resistance of the muscarinic agonist-induced contraction to β -adrenoceptor agonists is importantly determined by its efficacy to induce PI metabolism and subsequent changes in $[\text{Ca}^{2+}]_i$ (29).

Since β -adrenoceptor-mediated inhibition of methacholine- and histamine-induced Ca^{2+} responses as well as the forskolin- and 8-Br-cAMP-mediated inhibitions of methacholine-induced Ca^{2+} responses were not associated with significant inhibitory effects of PI metabolism, it can be concluded that cAMP-activated protein kinase A (PKA) is mainly effective at a different level of the Ca^{2+} -homeostasis in BTSM. Possible targets for PKA could be the IP_3 -receptor, causing a reduced Ca^{2+} release from the endoplasmic reticulum, as well as Ca-ATP-ases in the endoplasmic reticulum and plasma membrane, causing enhanced Ca^{2+} transport to intra- and extracellular compartments, respectively (see ref. 29).

Very surprisingly, in the BTSM cells we found that the inhibition of methacholine-induced Ca^{2+} influx by isoprenaline, but not by forskolin or 8-Br-cAMP, was considerably smaller when the β -agonist was added during the sustained phase of Ca^{2+} influx than when added before the contractile agonist, again illustrating that muscarinic receptor activation may cause β -adrenoceptor uncoupling. In accordance with the functional experiments described above, the reduced effectiveness of isoprenaline could be reversed by GF 109203X (unpublished), indicating that PKC is involved.

Role of muscarinic M_2 receptor stimulation in the functional antagonism of airway smooth muscle contraction by β -adrenoceptor agonists. Studies on the functional role of M_2 receptors in airway smooth muscle have been guided by the observation that these receptors inhibit adenylyl cyclase activity and may thus attenuate β -adrenoceptor-mediated relaxation. Thus, muscarinic M_2 receptor-mediated inhibition of adenylyl cyclase (as well as potassium channel closure) could contribute to the phenomenon that isoprenaline-induced relaxation of cholinergic tone is hampered compared to similar contraction levels of histamine, and that muscarinic agonists promote a larger shift of isoprenaline potency than histamine at a given level of IP accumulation (33).

In canine, rabbit and guinea pig trachea precontracted with muscarinic agonists, selective blockade of M_2 receptors or inactivation of G_i enhanced the relaxant potency of isoprenaline. However, the effects were relatively small compared to the large changes in isoprenaline relaxant potency that can be obtained in these tissues as a result of varying cholinergic contraction levels (reviewed in ref. 2). Moreover, in guinea pig trachea such role for muscarinic M_2 receptors was found completely absent when gallamine was used as the M_2 -selective antagonist (36). In BTSM, we observed highly significant correlations between the decrease of both pD_2 and E_{max} values of isoprenaline-induced relaxation and inhibition of adenylyl cyclase at increasing concentrations of methacholine, suggesting a possible role for muscarinic M_2 receptor-mediated inhibition of adenylyl cyclase in the decrease of β -adrenergic relaxability. However, quantitatively similar relationships of the pD_2 and E_{max} values with much lower levels of IP production were observed, indicating a more prominent role of PI metabolism than of G_i -mediated adenylyl cyclase inhibition

in the functional antagonism (37). This was supported by the observation that isoprenaline-induced relaxation of cholinergic tone was enhanced by the M_2 -selective antagonists gallamine and AF-DX 116, and by the M_1 and M_3 receptor-selective antagonists pirenzepine and hexahydrosiladifenidol, which could entirely be explained by small changes in initial contractile tone in the presence of these antagonists (38). As in BTSM, a role for M_2 receptors in functional antagonism in human bronchial smooth muscle appears to be absent, as indicated by identical relationships between IP production and isoprenaline-induced relaxation for both methacholine- and histamine-induced tones (25) and the observation that β -adrenergic relaxation of carbachol-induced contraction is not potentiated by methoctramine (39).

Although, depending on the species under investigation, cross-talk between muscarinic M_2 receptor and β -adrenoceptor function appears to play only a minor, if any, role in the functional antagonism of cholinergic contraction by β -agonists, such a role may become apparent in pathophysiological conditions, such as allergic asthma. Thus, very recently it has been demonstrated that passive sensitization with human atopic serum as well as incubation with inflammatory cytokines, such as IL-1 β and TNF- α , may cause attenuated β -adrenoceptor-mediated rabbit tracheal smooth muscle relaxation due to enhanced muscarinic M_2 receptor coupled activation of G_i , presumably by enhanced expression of the $G_{i\alpha 2}$ and/or $G_{i\alpha 3}$ subunits (see ref. 40).

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MUSCARINIC RECEPTOR SUBTYPE SELECTIVE TOXINS

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Summary

The muscarinic acetylcholine receptors are monomeric proteins with seven hydrophobic, membrane spanning helices, and share a common evolutionary origin with the other members of the superfamily of membrane proteins known as seven-helix receptors. The amino acid sequences of five different muscarinic acetylcholine receptors, called m1, m2, m3, m4 and m5 have been determined. The five subtypes are expressed to different extent in different tissues. A large number of low molecular ligands for muscarinic receptors are known, but they bind to all five subtypes of receptors and only a few of them have a slightly higher (five-six fold) affinity for one of the subtypes, e.g. pirenzepine for M₁ (1) and triptamine for M₂ receptors (2). Several neurotoxins have been isolated from snake venoms and used as pharmacological tools. Mambas, African snakes of genus *Dendroaspis*, have toxins that recognize muscarinic receptors and some of these muscarinic toxins are the most selective ligands for M₁ and M₄ receptors known to date.

Key Words: muscarinic toxins, acetylcholine muscarinic receptors, Alzheimer's disease, amino acid

Structure of muscarinic toxins

Eight muscarinic toxins have been isolated from the venom of *Dendroaspis angusticeps* (Eastern green mamba) and three from the venom of *D. polylepis* (Black mamba) by gel filtration, ion exchange chromatography and reverse phase HPLC (3-6) or by gel filtration and reversed phase HPLC (7). The green mamba toxins are called MT-1, 2,...MT-7 according to the order in which they were discovered or m1-toxin (7) which refers to subtype selectivity of the toxin. The black mamba toxins are called MT- α , β and γ (6) which also indicates the order of discovery.

Nine toxins have been sequenced by protein chemical methods, the sequence of one of the toxins has also been deduced from the cDNA nucleotide sequence (8). The toxins consist of 64-66 amino acids and four disulphides. The three-dimensional structure of the toxin MT-2 has been elucidated (9). Four peptide loops are formed by the disulphides. Three loops (I, II and III) protrude like the three middle fingers of a hand and the disulphides are in the palm of the hand. Many other snake toxins have a similar three-finger structure such as a-neurotoxins (bind to nicotinic acetylcholine receptors), cardiotoxins (increase permeability of membranes), fasciculins (inhibitors of acetylcholinesterase), calciseptine (blocker of L-type Ca-channels) and mambin (inhibitor of platelet aggregation).

Receptor affinity of muscarinic toxins

The toxins were tested on cloned human receptors expressed in chinese hamster ovary cells. The toxin concentration that caused 50% inhibition (IC₅₀) of the ³H-N-methylscopolamine (³H-NMS) binding was determined. The K_i values were calculated from the Cheng-Prusoff

formula (10): $K_i = IC_{50} / (1 + [L]/K_d)$, where $[L]$ and K_d are concentration and dissociation constant of 3H -NMS, respectively.

MT-3 displayed high affinity ($pK_i = 8.7$) for the m_4 receptor which was 40 times higher than for the m_1 receptor ($pK_i = 7.11$) (Table I). The Hill coefficients are close to 1, indicating binding to a single site. For comparison, the affinity values were reported for two compounds, pirenzepine and himbacine, that have been used as pharmacological tools to distinguish M_1 and M_4 receptors (4). It is evident that MT-3 is considerably more selective for one receptor than pirenzepine and himbacine.

Table I

Affinities of muscarinic toxins and other compounds to cloned human acetylcholine muscarinic receptors expressed in Chinese hamster ovary cells

Toxin	Receptor subtype: affinity (K_i) in nM				
	m1	m2	m3	m4	m5
<i>D. angusticeps</i>					
MT-1 (n= 7) ¹	49	>1000	>1000	58	>1000
MT-2 (n= 3) ²	1500	>2000	>2000	760	>2000
MT-3 (n= 4)	78	>1000	>1000	2	>1000
MT-4 (n= 3)	62	>1000	>1000	87	>1000
MT-5 (n= 3)	180	>1000	>1000	540	>1000
MT-6 (n= 1)	190	>425	>425	3.6	>425
m1-toxin ³	0.1	NB	NB	8	NB
MT-7 (n= 3)	0.2	>2000	>2000	>2000	>2000
<i>D. polylepis</i>					
MT- α (n= 3)	23	44	3	5	8
MT- β (n= 3) ⁴	>1000	>2000	140	120	350
Pirenzepine (n=4) ⁵	11	470	220	50	130
Himbacine (n=4) ⁵	210	12	140	13	4500

Values are means of n independent determinations.

- 1) Other values : $K_i = 45$ nM for m1, 72 nM for m4 (11-13). ^{125}I -MT-1 binds only to m1 receptors with a K_d of 21 nM (14).
- 2) The high K_i values most likely indicate that the toxin was not fully active. MT2 is easily inactivated probably due to hydrolysis of the particularly labile peptide bond Asp53-Pro54 (15). A fully active MT2 has the same affinity as MT-1, since a K_d of 17 nM has been reported for ^{125}I -MT2(16). ^{125}I -MT2 binds only to M_1 receptors (17).
- 3) Calculated from ref. 7, Fig.11 assuming 95% inactivation of the toxin. NB= no binding.
- 4) Probably partially inactivated preparation. 5) see reference 4

MT-1 and MT-4 can not distinguish between m1 and m4. The same is true for MT-5 although it has a 3 times higher affinity for m1 compared to m4 (Table 1). Due to limited amount of toxin available MT-6 was assayed only once but it seems to have the same selectivity as MT-3.

Muscarinic receptor subtypes in peripheral blood lymphocytes

The presence of a muscarinic receptor subtype in lymphocytes was reported by Adem et al., (1985)(18). Recently Costa et al., (1995) (19) and Hellström-Lindahl and Nordberg, (1996) (20) using RT-PCR technique demonstrated that lymphocytes express mRNAs for the m3, m4 and m5 subtypes. To investigate if lymphocytes have M₄ binding sites we used [¹²⁵I]-MT-3 as a ligand. At a concentration of 13 nM, [¹²⁵I]-MT-3 was bound to 33±5.5 fmol/mg (n=6) protein M₄ binding sites in lymphocytes indicating the presence of M₄ receptors in these cells.

Distribution of M₁ and M₄ muscarinic receptor subtypes in brain

Although the binding affinity of MT-1 for the ³H-NMS binding site at the M₁ and M₄ receptors does not differ very much, iodinated MT-1 is selective for M₁ than M₄ (21, 22). To further explore the issue of selectivity of ¹²⁵I-MT-1 we compared the distribution of ³H-pirenzepine (5 nM) and ¹²⁵I-MT-1 binding sites in consecutive sections of rat brain. The overall autoradiographic distribution of ¹²⁵I-MT-1 binding sites in the rat brain is similar but not identical to that seen using ³H-pirenzepine possibly because pirenzepine labels some M₄ receptors in addition to M₁ receptors (22).

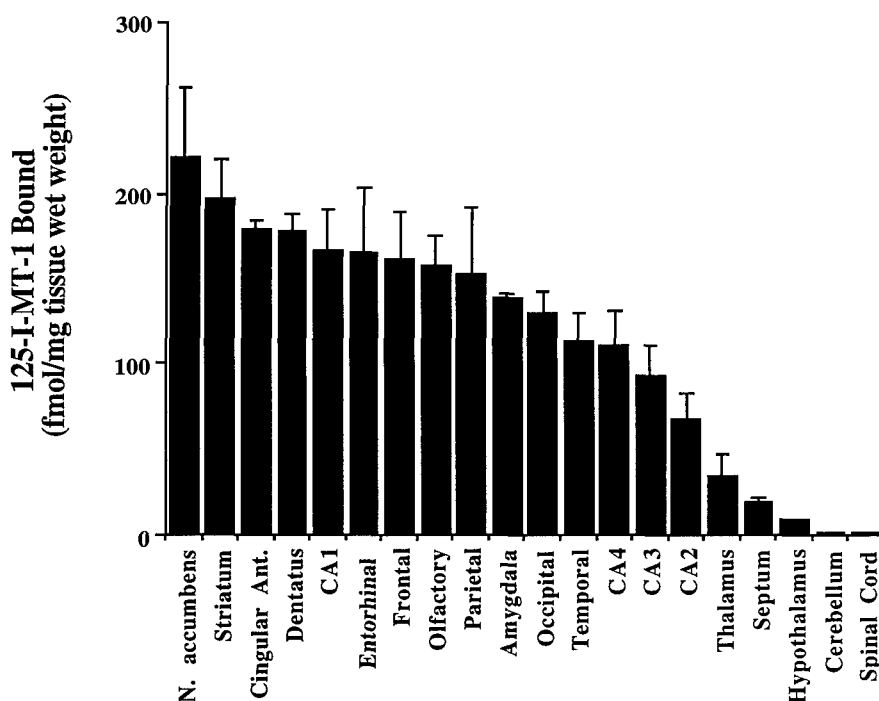


Fig.1

Distribution of ¹²⁵I-MT-3 binding sites in selected areas of the rat brain.

The nucleus accumbens, striatum and dentate gyrus showed highest labelling by ^{125}I -MT-1 (Fig. 1). High densities of ^{125}I -MT-1 binding sites are located in the CA1 region of the hippocampus, frontal and parietal cortices whereas moderate densities of binding sites were seen in temporal cortex, and hippocampal subregions CA2, CA3, and CA4. The pons, cerebellum and spinal cord showed extremely low densities of binding sites. The presence of high levels of ^{125}I -MT-1 binding sites in the striatum, neocortex and hippocampus is in agreement with the reported findings which show M_1 receptor protein enrichment, using subtype selective antibodies, in these areas (23, 24). Thus our results demonstrate that ^{125}I -MT-1 binds to M_1 muscarinic receptors and that ^{125}I -MT-1 is a much more selective ligand than pirenzepine for binding studies of M_1 muscarinic receptors.

The regional distribution of [^{125}I]-MT-3 binding sites varies in different areas of rat brain (Fig.2). Quantitative measurement of [^{125}I]-MT-3 autoradiography in rat brain sections indicated a high labelling in the islets of Calleja, striatum, and accumbens; a moderate labelling in the cortex, lateral geniculate, and thalamus. Low labelling was obtained in the amygdala, hippocampus and hypothalamus whereas very low labelling was obtained in the spinal cord. However, our results show that the spinal cord has higher levels of M_4 than M_1 receptors. The presence of high levels of M_4 receptors in the islets of Calleja and neostriatum obtained by MT-3 binding is in agreement with the findings of Levey et al.(1991) (23) who reported M_4 receptor protein enrichment, using subtype selective antibodies, in these areas. Taken together these results indicate that the M_4 muscarinic receptor subtype is highly localized in subcortical areas which project to the cortex.

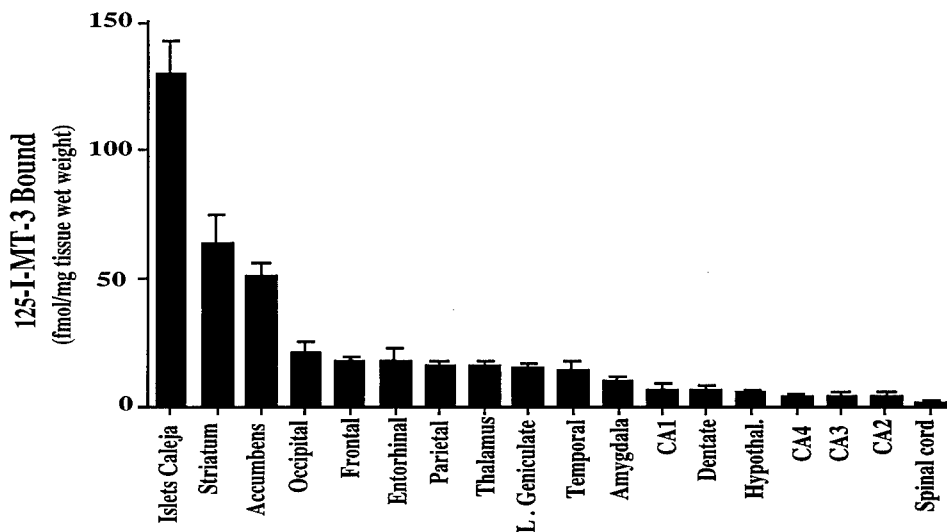


Fig.2

Distribution of ^{125}I -MT-3 binding sites in selected areas of the rat brain.

The distribution of ^{125}I -MT-1 binding sites in the rat brain were different from those of ^{125}I -MT-3. The difference in selectivity of these toxins can be used to study how the different muscarinic receptor subtypes are affected in disease states. In this respect, we studied the distribution of ^{125}I -MT-1 and ^{125}I -MT-3 binding in human hippocampus from Alzheimer patients and age matched controls. A significant reduction was obtained in the M_4 but not in the M_1 receptors of the dentate gyrus of Alzheimer patients compared to controls (Fig.3).

Structure-activity relationship

MT- α and MT-4 differ only by three amino acids, but they have a different subtype specificity. MT- α has a tripeptide Leu-Asn-His, 31-33, and binds with high affinity for all subtypes. MT-4 has instead the tripeptide Ile-Val-Pro, 31-33, and recognizes only m1 and m4 receptors (Tables I and II). Another example of mutations of a few amino acids changing the subtype specificity is found in MT-7 and m1-toxin. MT-7 has a dipeptide Trp-Gln, 28-29, and binds only to m1 receptors. m1 toxin has instead the dipeptide Trp-Gln, 28-29, and lacks Lys 65. The toxin binds to m1 receptors with about the same affinity as MT-7 but binds also to m4 receptors. In one case a single amino acid mutation changes the specificity. MT-5 with Lys 48 binds to m1 and m4 and MT- β with Ile 48 to m3, m4 and m5 receptors.

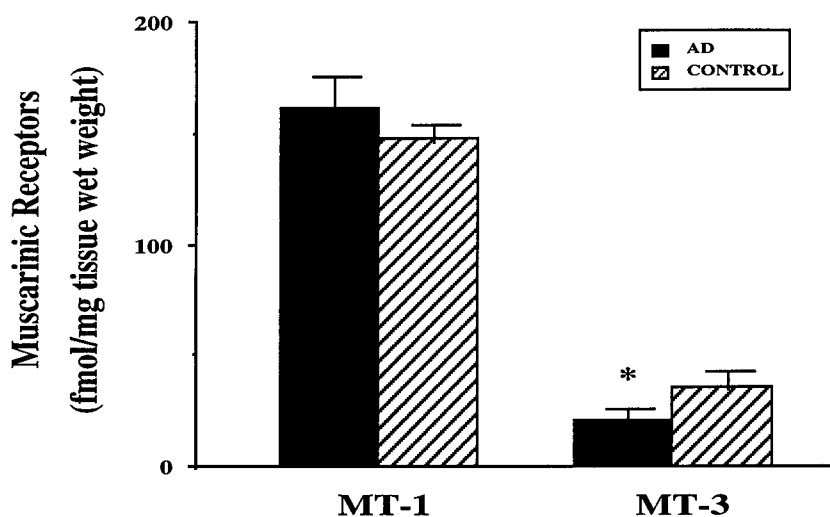


Fig.3

Muscarinic receptor subtype changes in Alzheimer's disease (controls $n=4$, mean age \pm sem= 81 ± 1.2 ; Alzheimer patients $n=8$, mean age \pm sem= 81 ± 2)

Another remarkable feature is that iodination of MT-1 and MT-2 makes the toxins more specific. Native toxins bind to M_1 and M_4 and after iodination only to M_1 . This has been demonstrated for MT-1 by several independent ways. Inhibition of ^{125}I -MT-1 binding by pirenzepine gave a K_i of 8 nM (14) in good agreement with the value 6.3 nM given in ref.1. ^{125}I -MT-1 binds to a slightly smaller area in rat brain than low concentration (5 nM) of ^3H -pirenzepine (22). A similar binding was also observed with ^{125}I -MT-2 and 2.5 nM ^3H -pirenzepine (17). At these concentrations pirenzepine should label mostly M_1 receptors. Finally, ^{125}I -MT-1 has been shown to bind only to cloned m1 receptors (21).

The site of iodination has not been determined. MT-1 and MT-2 have three and two tyrosines, respectively, and the only common one is tyrosine 30 which is invariant in all muscarinic toxins. Iodination produces the same change in specificity in both toxins, and therefore, the same site may have been modified. As seen from the three dimensional structure of MT-2, tyrosine 30 has a very exposed position (9) and should be very reactive. Iodination (by

chloramine T method) labelled only tyrosine and was carried out for a short time (30 - 60 sec) using a high excess of toxin. Only 1-2 % of the molecules were iodinated and only one iodine atom should have been incorporated into the most reactive tyrosine, which probably is tyrosine 30. Incorporation of a bulky iodine atom into a tyrosine residue evidently makes MT-1 and MT-2 incapable of binding to m4 receptors.

Table II

Invariant amino acids are marked with two dots. The other sequences are from the following sources; MT-1 (13), MT-2 (8, 25), MT-3 (4), MT-4 (5), MT-6, MT-7 (unpublished), m1-toxin (7), MT- α and MT- β (6).

	1	10	20	30	40	50	60	66
MT1	LTCVTSKSI	FGITTENC	PDGQNL	CFKKWY	IVPRYS	DITWGA	ATCPKPT	NVRETI
MT2	LTCVTTKSI	GGVTTED	CPAGQNV	CFKRWH	YVTPKN	YDIKGA	ATCPKVD	NN-DPI
MT3	LTCVTKN	TIFGITT	ENC	PAGQNL	CFKRWH	YVIPRY	TEITRG	CAATCPI
MT4	LTCVTSKSI	FGITTENC	PDGQNL	CFKKWY	IVPRYS	DITWGA	ATCPKPT	NVRETI
MT5	LTCVTSKSI	FGITTED	CPDGQNL	CFKRRH	YVVPKI	YDITRG	CVATCP	KPENY-
MT7	LTCVKNSI	WFPTSED	CPDGQNL	CFKRQY	ISPRMY	DFTRG	CAATCP	KAEYR-
m1	LTCVKNSI	WFPTSED	CPDGQNL	CFKRWH	YISPRMY	DFTRG	CAATCP	KAEYR-
MT α	LTCVTSKSI	FGITTENC	PDGQNL	CFKKWY	LNHRY	SDITWG	CAATCP	KPTNVRE
MT β	LTCVTSKSI	FGITTED	CPDGQNL	CFKRRH	YVVPKI	YDITRG	CVATCPI	PENY-

Mutations and iodination that change subtype specificity probably occur at sites which participate in the binding to receptors. The corresponding amino acids are located in the second loop (Cys 46 - Cys 42) and in the third loop (Cys 46 - Cys 58), lys/Ile 48. The second loop is very protruding with a cationic residue, Lys or Arg 34, at the tip (9). Muscarinic receptors consist of six extracellular, seven transmembrane and six intracellular domains. According to models of muscarinic receptors (26-29) the transmembrane parts form a deep cleft with an aspartic acid residue present in the third transmembrane region of all muscarinic receptors. Mutagenesis studies indicate that this aspartic acid residue is essential for binding of acetylcholine.

The second loop may penetrate the cleft and Lys/Arg 34 interact with the conserved aspartic and the first and third loops would be in contact with the extracellular domains (9). This model may also explain the better selectivity of the muscarinic toxins as compared to the low molecular ligands. Due to their small size, the latter ligands will probably interact only with amino acids in the cleft.

With the exception of the first transmembrane region, which has only nine conserved residues out of 24, the transmembrane regions are rather similar in all subtypes. This may partially explain why low molecular weight ligands do not discriminate well between the subtypes. The better selectivity of the snake toxins may depend on two factors. First, they discriminate better between the differences in the clefts of the various subtypes. The residues 28-29, 30 and 31-

33 responsible for differences in selectivity are close to the cationic group at position 34, and the different selectivities should be due to different interactions of these residues with amino acids in the cleft. Secondly, the toxins probably interact via the loops I and III with the extracellular domains which are variable both in length and composition.

An alternative view is the possibility that the second loop of these toxins bind to allosteric sites and not to the acetylcholine site in the second transmembrane region and one of the loops (loop I or loop III) may interact with the first transmembrane region and the other with the extracellular domain of the receptor.

Functional effects of muscarinic toxins

MT-1 and MT-2 when injected into the dorsal hippocampus caused memory facilitation in an inhibitory avoidance learning task which was antagonized by the simultaneous injection of scopolamine (11). MT-1 and MT-2 acted like the relatively selective M₁ agonist McNA 343 in reducing the responses of rabbit vas deferens to nerve stimulation possibly by acting on presynaptic M₁ receptors on this preparation (30). MT-2 was shown to enhance carbachol-stimulated phosphatidylinositol turnover in homogenates of rat cerebral cortex (17). However, m1-toxin has been shown to block the effect of carbachol on phosphatidyl turnover in rat hippocampal slices (31). Recently, Olanas et al., (1996) (32) reported that MT-3 is a potent antagonist of the striatal muscarinic receptors coupled to inhibition of adenylyl cyclase activity. Moreover, MT-3 antagonized the acetylcholine inhibition of adenylyl cyclase with low affinity.

Concluding remarks

Mamba venoms contain several muscarinic receptor subtype selective toxins. The difference in selectivity of these toxins can be used to study how the different muscarinic receptor subtypes are affected in disease states. In this respect, using M₁ and M₄ subtype selective toxins, a significant reduction was obtained in the M₄ but not in the M₁ receptors of the dentate gyrus of Alzheimer patients compared to controls. These toxins (MT-1 and MT-3) and other subtype selective toxins in mamba venoms may provide information to new strategies in future drug design of M₁, M₄ and other receptor subtype selective drugs which might be effective in the treatment of diseases associated with dysfunction of the central or peripheral cholinergic system.

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PATHWAYS AND ROADBLOCKS IN MUSCARINIC RECEPTOR-MEDIATED GROWTH REGULATION

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Summary

In some cell systems muscarinic receptor stimulation can induce proliferation or transformation. This phenomenon is subtype-specific (only m1 and m3 receptors are effective) and cell type dependent. In 1321N1 astrocytoma cells activation of m3 receptors stimulates phospholipase C, but does not induce DNA synthesis. In contrast the thrombin receptor, which also couples to phospholipase C, is strongly mitogenic and induces AP-1-dependent gene expression. Various experimental findings indicate that this discrepancy is not due to muscarinic receptor desensitization or blockade of growth stimulatory pathways. Muscarinic receptor number may be limiting, in particular for receptor coupling to the pertussis toxin-insensitive G-protein G_{12} . This G-protein is required for thrombin-induced mitogenesis in 1321N1 cells and may couple selectively to the thrombin versus muscarinic receptor. In cardiomyocytes hypertrophic cell growth is induced by heterologously expressed m1 or m3 receptors but not by the endogenous m2 receptors. Studies using chimeric receptors confirm that induction of hypertrophy requires signalling through phospholipase C, but indicate that additional signals are needed to induce the morphological features of this response. We suggest that small G-proteins of the Rho subfamily, in addition to G_{12} , mediate growth responses to G-protein-coupled receptors.

Key Words: thrombin receptor, DNA synthesis, cardiomyocytes, G proteins, gene expression

Stimulation of receptor tyrosine kinases leads to the induction of cell proliferation and differentiation through a sequence of molecular events that have been extensively delineated. G-protein-coupled receptors have more recently been shown to also regulate cell growth. However, the molecular events linking G-protein-coupled receptors to cell growth are not well defined. Recent evidence indicates that G-protein-coupled receptors can activate some of the same signal transduction pathways first described for the receptor tyrosine kinases (1-3). An observation that may provide mechanistic insights is that only a subset of G-protein-coupled receptors are capable of inducing growth responses (4-8). Our work has focused on two model systems: the 1321N1 astrocytoma cell line, which undergoes DNA synthesis in response to thrombin receptor but not m3 muscarinic receptor stimulation, and the neonatal rat ventricular myocyte, in which features of cardiac hypertrophy are reproduced *in vitro* upon activation of α_1 -adrenergic receptors but not of the endogenous m2 muscarinic receptors. The studies described here explore the question of what determines the ability of muscarinic receptors to induce cell growth.

Muscarinic versus thrombin receptor effects on DNA synthesis and AP-1-mediated gene expression in 1321N1 astrocytoma cells.

mAChR- and thrombin receptor-mediated phospholipase signalling

The muscarinic receptor (mAChR) in 1321N1 cells was demonstrated in T.K. Harden's laboratory to lower cyclic AMP metabolism by a unique mechanism, independent of G_i -mediated adenylate cyclase inhibition (9). The mAChR in these cells regulate phosphoinositide hydrolysis and Ca^{++} mobilization (10,11) and were later shown to be predominantly of the m3 subtype (12), which couples to phospholipase C via $G\alpha_q$ (13). We further demonstrated regulation of phospholipase D, generation of diacylglycerol and activation of protein kinase C in response to mAChR stimulation in 1321N1 cells (14-16). The 1321N1 cell also expresses the G-protein-coupled thrombin receptor. Like the mAChR, the thrombin receptor regulates phospholipase C, Ca^{++} mobilization, phospholipase D and protein kinase C activity (6,14,17). The magnitude of these responses is comparable for thrombin receptor and m3 receptor activation (6).

mAChR and thrombin receptor effects on AP-1-mediated gene expression and DNA synthesis

In subsequent studies we found that mAChR stimulation increased *c-fos* and *c-jun* mRNA expression (18). Since *c-fos* and *c-jun* encode proteins that form the transcription factor AP-1 (19), we hypothesized that mAChR stimulation would induce AP-1-dependent gene expression. Unexpectedly, an AP-1-sensitive luciferase reporter gene (2XTRE-luciferase) transiently transfected into 1321N1 cells, was at best modestly activated in response to carbachol. In contrast, thrombin induced a robust increase in 2XTRE-luciferase gene expression. (20). Ashkenazi *et al.* demonstrated carbachol-stimulated [3H]thymidine incorporation into DNA in 1321N1 cells (4). However using BrdU incorporation as an index of DNA synthesis we found that whereas thrombin increased the proportion of BrdU staining cells from 10% to ~50%, carbachol had no effect. Using [3H]thymidine incorporation we confirmed published observations that carbachol increased DNA synthesis (4,21). The effect of carbachol was, however, very modest compared to that of thrombin (6). Thus, as also shown for CCL 39 fibroblasts (5), activation of phospholipase C-coupled pathways in is not sufficient to induce mitogenic responses.

Hypotheses for failure of m3 mAChR to regulate AP-1-mediated gene expression and DNA synthesis

We examined several possible explanations for the divergent effects of the m3 and thrombin receptor on growth-associated responses in 1321N1 cells. First, we considered that there might be rapid desensitization of mAChR mediated responses. However we found that inositol phosphate formation in response to carbachol is sustained (22) while thrombin-stimulated phosphoinositide hydrolysis and calcium mobilization desensitize more rapidly (17). Thus, thrombin signalling, rather than carbachol-induced responses, should be limited by desensitization. The possibility that carbachol might inhibit growth factor signalling pathways in 1321N1 cells was suggested by data demonstrating that activation of the MAP kinase cascade by EGF was inhibited by simultaneous m1 receptor stimulation (23). However, we found that carbachol did not inhibit growth factor (thrombin, bFGF, EGF)-stimulated DNA synthesis in 1321N1 cells (6).

Although m3 receptors on 1321N1 cells couple efficiently to phospholipase C activation and generation of its known second messengers, the number of mAChR of these cells is fairly low (<50 fmol/mg protein). Thus, a reasonable explanation for the disparate growth effects of the thrombin and muscarinic receptors could be that m3 receptor number is limiting for regulation of growth responses. We have not been successful at creating stable transfectants of 1321N1 cells expressing higher numbers of m3 receptors. However, J. Schachter in the Harden laboratory recently isolated spontaneously appearing subclones of 1321N1 cells with an enhanced phosphoinositide response to carbachol. These cells (termed 132M cells) have an increased number of mAChR (Fig. 1). Despite the higher receptor expression and associated enhancement of phosphoinositide hydrolysis, carbachol still failed to elicit greater DNA synthesis (Fig. 1). While these data suggest that mAChR

number is not limiting, a receptor density far higher than that achieved here (~400 fmol/mg protein) was required for carbachol to regulate proliferation in NIH3T3 cells expressing m1 or m3 receptors (8). Thus it may well be that the m1 and m3 receptors couple efficiently to growth regulatory pathways only when they are overexpressed.

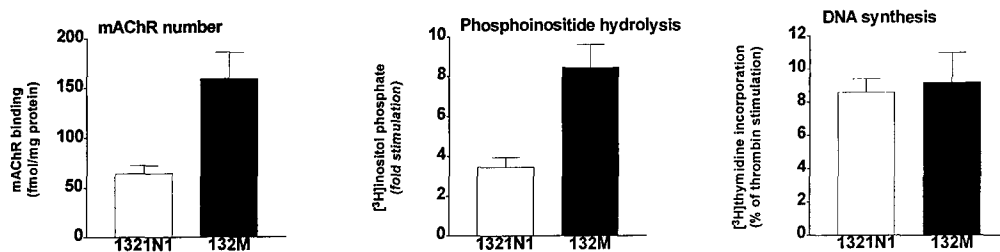


Fig. 1. Carbachol fails to stimulate DNA synthesis in 132M cells despite increased mACHR number and enhanced carbachol-stimulated phosphoinositide hydrolysis.

Muscarinic receptor number (left panel) was estimated based on the specific binding of [³H]N-methyl-quinuclidinylbenzylate to whole cells using a concentration of ligand (2 nM) approximately 10 times its K_d . Phosphoinositide hydrolysis (center panel) was assayed in cells labelled overnight with [³H]inositol and then treated with 500 μ M carbachol for 20 minutes in the presence of lithium chloride. Total inositol phosphates were collected and counted. DNA synthesis (right panel) was determined by labelling cells with [³H]thymidine for the last 6 hr of a 24 hr exposure to thrombin (0.5 units/ml), carbachol (500 μ M), or vehicle. The response to carbachol is expressed relative to that for thrombin. All data are mean \pm SEM from 2 experiments done in triplicate.

Selective coupling of the thrombin versus m3 receptor to G_{12}

The studies discussed above suggested that receptor coupling to G_q /phospholipase C was insufficient to generate growth responses. Thrombin receptor coupling to G_i and G_s does not mediate DNA synthesis or 2XTRE-luciferase gene expression since these responses are pertussis toxin-insensitive (6). We examined the role of G_{12} , a newly discovered pertussis toxin-insensitive G-protein (24,25), in thrombin action. The direct effectors of G_{12} are unknown, but this G-protein was originally identified as an oncogene in transformed cells (26), and has been demonstrated to have growth regulatory properties in other cell systems (25). In platelets, thrombin receptor stimulation activates G_{12} as assessed by increased GTP-azidoanilide binding (27). We tested the possible role of G_{12} in thrombin-induced mitogenesis by microinjecting 1321N1 cells with an antibody to G_{12} prior to stimulation with thrombin. BrdU incorporation was subsequently assessed in control (IgG)- and $G\alpha_{12}$ antibody-injected cells. The $G\alpha_{12}$ antibody was shown to specifically inhibit thrombin-stimulated DNA synthesis (28).

In related studies we transiently expressed a constitutively activated form of $G\alpha_{12}$ in 1321N1 cells (or in HEK293 or COS-7 cells to achieve higher transfection efficiency). These studies demonstrated that activated $G\alpha_{12}$ increased tyrosine phosphorylation of the adaptor protein Shc (29), increased Ras-GTP binding (30), activated the cJun N-terminal kinase (JNK) (30), and increased 2XTRE-luciferase reporter gene expression (28). Each of these responses was also elicited by thrombin but none were consistently induced by a constitutively activated form of $G\alpha_q$. These data suggested that thrombin receptor coupling to G_{12} was responsible for its ability to regulate AP-1-

mediated gene expression and DNA synthesis. The distinct effects of m3 and thrombin receptor activation could therefore result from selective activation of G_{12} by the thrombin receptor. Coupling of the m3 and thrombin receptors to G_{12} was examined by expressing cDNA encoding these receptors in COS-7 cells, along with cDNA for wild type $G\alpha_{12}$ or $G\alpha_q$. An AP-1- sensitive luciferase reporter gene was induced when the appropriate agonist was added to cells expressing either m3 muscarinic or thrombin receptors. The response to thrombin receptor activation was markedly enhanced in cells co-transfected with the $G\alpha_{12}$ (but not the G_q) expression plasmid (6). Interestingly there was no potentiation of the m3 receptor-mediated response by $G\alpha_{12}$, suggesting that the m3 receptor does not effectively couple to this G-protein to regulate downstream effectors.

Based on the data summarized above we suggest that the m3 receptor differs from the thrombin receptor in that the latter couples more efficiently to G_{12} , and thus to signalling cascades required for growth regulation. Studies using chimeric m3: thrombin receptors may provide further insight into the possibility of differential coupling, as will defining the regulators and effectors of G_{12} action.

Hypertrophic growth and cardiac gene expression in ventricular myocytes

Characteristics of G-protein-coupled receptor-simulated growth responses

Ventricular myocytes isolated from neonatal rats are terminally differentiated and therefore do not divide in response to mitogens. However, a highly characteristic form of cell growth which resembles hypertrophy can be induced by activation of certain G-protein-coupled receptors. Stimulation of the α_1 -adrenergic receptor (α_1 -AdR) with phenylephrine (PE) leads to increases in myocyte cell size and myofilament organization, re-expression of embryonic genes such as that for atrial natriuretic factor (ANF), and upregulation of genes encoding contractile proteins such as myosin light chain-2 (31). The molecular signals regulating this complex set of responses are not completely understood but one key player is the small G-protein Ras. Microinjection of a dominant interfering Ras expression plasmid blocks PE-induced ANF expression, thereby demonstrating a requirement for Ras in α_1 -AdR effects (32). Additionally, overexpression of activated Ras in transgenic mice can induce hypertrophy *in vivo* (33). The signalling cascade initiated by Ras activation leads to phosphorylation of the extracellular signal-regulated kinases (ERKs), implicated in proliferation and differentiation (34). ERKs can be activated by both the m1 and m2 receptors, as well as by α_1 -AdR, implicating these receptors in growth regulation (35-38). We compared the ability of the cardiac mAChR and α_1 -AdR to stimulate ERK and induce growth responses in myocytes.

Effects of mAChR activation on ERK activity and hypertrophic responses in rat ventricular myocytes

ERK activation was examined in neonatal rat ventricular cardiomyocytes stimulated with PE to activate α_1 -AdR, or carbachol to activate mAChR (7). These studies demonstrated that carbachol, like PE, increased the activity of both ERK1 and ERK2. The stimulatory effects of PE and carbachol on ERK activity were comparable, both increasing the phosphorylation of myelin basic protein (MBP) ~ 2-4 fold. Additionally, ERK activation occurred with similar kinetics in response to activation of either the mAChR or the α_1 -AdR in cardiac myocytes (7). The downstream responses characteristic of hypertrophic growth were then compared. We examined gene expression by transient transfection of ANF- or MLC-2-luciferase reporter genes, changes in cell morphology by staining myofilaments with antibody to MLC-2, and increases in ANF protein using an antibody to ANF. In contrast to PE carbachol did not increase ANF or MLC-2 luciferase gene expression, cell size, organization of myofilaments or ANF protein expression (7). Thus ERK activation alone is insufficient to elicit the characteristic growth responses seen with α_1 -AdR activation.

The predominant subtype of mAChR in the heart is the m2 receptor, which couples to G_i to

inhibit adenylate cyclase (see (39)). Published studies demonstrating ERK activation by m2 receptors suggest that the enzyme is activated by release of $\beta\gamma$ subunits from G_i (36,40). Pertussis toxin blocks agonist-induced release of $\beta\gamma$ subunits from G_i and can therefore prevent $\beta\gamma$ -induced ERK activation. We treated cardiomyocytes with pertussis toxin (PTX) and examined ERK activation by carbachol and PE (Fig 2). Carbachol failed to increase ERK activity in pertussis toxin-treated cells, implicating a G_i -dependent pathway in ERK activation. In contrast, PE-induced ERK activation was pertussis toxin-insensitive, consistent with the coupling of the α_1 -AdR to G_q rather than G_i . A similar observation was recently made by Sugden's laboratory (41).

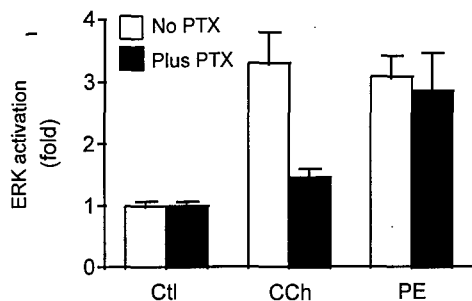


Fig. 2. Carbachol stimulates ERK in myocytes through a pertussis toxin-sensitive pathway.

Rat ventricular myocytes were cultured overnight in the absence or presence of 100 ng/ml pertussis toxin (PTX). Cells were then stimulated for 5 min with carbachol (CCh) or phenylephrine (PE), lysed, and lysates immunoprecipitated with ERK1 antibody. Activity of the immobilized enzyme was determined by measuring *in vitro* phosphorylation of myelin basic protein during a 12 min incubation at 30°C in the presence of γ [32 P]ATP.

Involvement of G_q - but not G_i -mediated pathways in induction of hypertrophic responses

The cardiac m2 receptor inhibits adenylate cyclase and, as demonstrated above, activates ERK through its interaction with G_i . Activation of the cardiac mAChR causes little phosphoinositide hydrolysis (42), consistent with its low efficacy for interaction with G_{α_q} . We tested the hypothesis that the activation of m2 receptors would effect changes in cardiac cell growth if this receptor could couple to phospholipase C by transfecting cardiac myocytes with a chimeric G-protein α -subunit (q_i , provided by B. Conklin) in which the N terminal 5 amino acids from G_{α_i} replaced those of G_{α_q} (43). Expression of this chimeric G-protein along with the ANF-luciferase reporter gene rendered the cells responsive to carbachol (Adams et al, in preparation) suggesting that an m2 receptor coupled to phospholipase C can elicit growth responses. We also transiently expressed mAChR subtypes that couple to G_q . The cDNA for the human m1 receptor (provided by Dr Eliot Ross) was transfected into myocytes and cells stimulated with carbachol. Carbachol elicited, through a pirenzepine-sensitive receptor, increases in ANF- and MLC-2-luciferase reporter gene expression, as well as an increase in ANF protein expression (42). Similar results were obtained in studies using cDNA for rat m3 receptors provided by Dr. Jurgen Wess (44).

To examine myofilament organization, myocytes were cotransfected with RSV-luciferase along with the mAChR expression plasmid and identified by immunostaining for luciferase. Phalloidin was used to stain actin myofilaments. Cells identified as luciferase-positive (which should also be expressing the transfected mAChR) had organized myofilaments when cultured in the presence of carbachol (Fig. 3). Myofilament organization was not observed in cells grown in the absence of carbachol, or in cells transfected with backbone vector. However, more than 50% of the cells transfected with m1 or m3 receptor cDNA showed organized myofilaments when stimulated with carbachol.

We next asked whether similar structural determinants were required for mAChR coupling to effectors involved in cardiac hypertrophy and to phospholipase C. For these studies we transfected chimeric or mutant receptors shown to have altered abilities to regulate formation of inositol phosphates. A chimeric receptor in which the N-terminal 21 amino acids from the i_3 loop

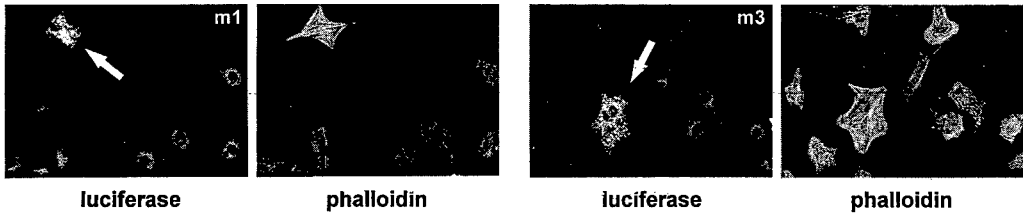


Fig. 3. Myocytes transfected with m1 or m3 receptor cDNA display increased cell size and organized myofilaments when stimulated with carbachol. Myocytes were co-transfected with RSV-luciferase and the indicated muscarinic receptor cDNA. Following 48 hr incubation in the presence of carbachol, cells were fixed and stained with an FITC-labelled antibody to luciferase and with rhodamine-conjugated phalloidin. A luciferase-positive cell in each field is identified by the arrow.

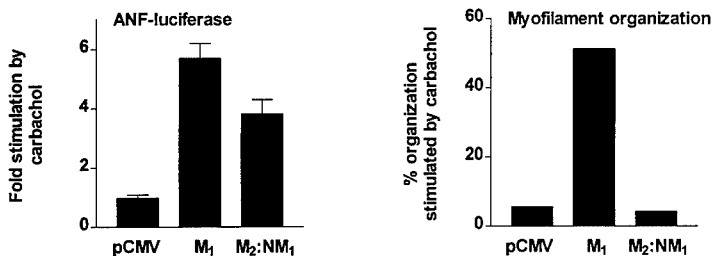


Fig. 4. Dissociation between the ability of the chimeric M₂:NM₁ receptor to stimulate ANF-luciferase expression and induce myofilament organization. Myocytes were transfected with the indicated receptors cDNAs and either the ANF-luciferase reporter gene or RSV-luciferase. Following a 48 hr incubation in the absence or presence of carbachol, cells were either lysed and luciferase activity measured (left) or were fixed, stained and analyzed for myofilament organization (right) as described for Fig. 3.

of the m1 receptor were replaced with the corresponding region from the m2 receptor failed to transduce signals leading to ANF gene expression (42). A similar substitution in the m3 mAChR also abolished coupling to ANF gene expression (44). Deletion of amino acids 252-259 or exchange of critical amino acids in this region was shown by Wess and colleagues to inhibit phospholipase C activation (45). These mutations also markedly attenuated the capacity of the transfected receptor to regulate ANF expression (44). However, a chimeric m2 receptor containing 21 amino acids from the N-terminal of the i3 loop of the m1 receptor (M₂:NM₁) was able to regulate ANF gene expression in response to carbachol (42). This chimeric receptor has been shown to be as efficacious as the wild type m1 receptor for stimulating phosphoinositide hydrolysis in response to carbachol in COS cells (46). Thus the requirements for receptor coupling to pathways regulating myocyte cell growth parallel those for coupling to phospholipase C.

Coupling to Gq/phospholipase C: a necessary but insufficient stimulus for mAChR-mediated growth regulation

In our studies with the 1321N1 cells we concluded that regulation of phospholipase C and its downstream signals by the endogenous m3 receptor was not sufficient to activate AP-1-mediated gene expression or DNA synthesis. It also appears that receptor coupling to phospholipase C is not

sufficient to induce all features of cardiac hypertrophic growth. As demonstrated in Fig. 3, carbachol induces myofilament organization in cells expressing m1 or m3 receptors. However cells expressing the chimeric M_2 :NM₁ receptor, which supports activation of the ANF luciferase gene, do not exhibit increased myofilament organization in response to carbachol (Fig 4). Interestingly, our previous studies showed this chimeric receptor to activate ANF gene expression through a Ras-independent pathway, in contrast to the wild type m1 receptor or endogenous α_1 -AdR which showed Ras-dependent signalling (42). Taken together these data suggest that the minimal region of the receptor required for interaction with G_q and activation of phospholipase C is not sufficient to specify Ras activation or induce morphological responses. Morphological responses in other systems are known to depend on small G-proteins of the Rho family which interact with the cytoskeleton (47). Recent studies from our lab demonstrate that Rho is required for α_1 AdR-induced hypertrophic responses in cardiac myocytes (48). Thus additional regions of G-protein-coupled receptors may participate in activation of small G-proteins and subsequent cytoskeletal changes or kinase cascades that lead to growth responses.

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MUSCARINIC RECEPTORS INVOLVED IN HIPPOCAMPAL PLASTICITY

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Israel**Summary**

The cholinergic septohippocampal system has been associated with learning and memory, as evidenced by the severe loss of these functions in lesioned animals as well as in senile demented patients. In an attempt to comprehend the physiological basis of the cholinergic innervation for hippocampal functions, numerous studies employed the in-vitro hippocampal slice preparation and analyzed the consequences of exposing the cells to cholinergic ligands. Many effects of activating a cholinergic receptor in the hippocampus were thus described, including blockade of several types of potassium conductances, yet few of these effects are intuitively related to the involvement of the cholinergic system in hippocampal plasticity. An alternative approach involves focusing on the possible effect of low concentration of cholinergic ligands on reactivity of the hippocampus to afferent stimulation. We found two new actions of acetylcholine (ACh); The first one is a fast onset, short lived increase in cellular responses to activation of the N-methyl-D-aspartate (NMDA) receptor, and the second one is a slow onset, long lasting increase in reactivity to afferent stimulation, resembling that produced by a tetanic stimulation, which we called muscarinic long term potentiation (LTPm). The latter effect is mediated by a postsynaptic M2 receptor, and it shares several properties with the more familiar tetanic LTP. In addition, LTPm involves a rise of intracellular calcium concentration and an activation of both a tyrosine kinase and a serine/threonine kinase. Intuitively, LTPm is better related to hippocampal plasticity than the other reported effects of ACh in the hippocampus. Indeed, aged rats, which are cognitively impaired, lack LTPm while they do express other muscarinic actions. It is proposed that LTPm is an important link between the cholinergic action and function in the hippocampus.

Key Words: hippocampus, muscarinic long term potentiation (LTPm), muscarinic receptors

Neuromodulators, substances released from presynaptic terminals which by themselves do not produce fast synaptic actions, are able to modulate reactivity of affected neurons to stimulation of other afferents. This can be achieved by affecting ion channel kinetics, thereby changing excitability of the cell in general, by affecting release of the fast neurotransmitters, or via second messenger interactions with the fast postsynaptic neurotransmitter receptors. The intuitive significance of the neuromodulators becomes evident following selective lesions or drug treatment, resulting in severe loss of brain functions. Such is the case with acetylcholine (ACh), which is released from septohippocampal terminals and is assumed to play a major role in cognitive processes of hippocampal learning and memory (1-3). Despite extensive studies, the role of ACh at the cellular and molecular levels of these processes is still unclear. In the present review, we will describe some novel actions of ACh, which may be related to the putative role of ACh in LTP, learning and memory.

The cholinergic innervation of the hippocampus arises solely from the medial septum and diagonal band of Broca (4-6). These are heterogeneous nuclei which also contain GABAergic neurons, also shown to innervate the hippocampus. Early stimulation and recording studies, being unaware of the complexity of the septohippocampal pathway, are likely to have obtained erroneous results due to stimulation of GABAergic fibers to the hippocampus, along with the cholinergic fibers. The cholinergic fibers, while not as precisely localized as the fast excitatory fibers arising from the entorhinal cortex and the commissural association pathway, are not at all diffuse throughout the hippocampus- a high concentration of fibers are seen in stratum oriens of CA1 as well as the dentate hilus (4-6).

Cholinergic Actions in the Hippocampus

There are five known subtypes of the muscarinic receptor expressed in mammalian brain, M1-M5 (7,8). The main muscarinic receptor in the hippocampus, M1, is highly concentrated in stratum radiatum and oriens of CA1, whereas M2 receptor is high in stratum oriens. The differential distribution of M1 receptors and the cholinergic fibers, stained for acetylcholine esterase (AChE) is one of the classical cases of 'mismatch' of fibers/receptors, which has no simple explanation: Why is there such a high concentration of M1 receptors in an area which has few cholinergic fibers? The fact that M1 is a low affinity receptor, which may never 'see' ACh released from its terminals, due to a diffusion distance and a fast breakdown of ACh by AChE, certainly complicates interpretation of studies on the action of ACh or carbachol (CCh) applied by perfusion onto hippocampal slices, as described below.

ACh, acting on muscarinic receptors in the hippocampus causes blockade of several potassium currents including a voltage and calcium-gated potassium current which underlies the slow afterhyperpolarization (I_{AHP}), a depolarization evoked sustained K current (I_M), and a resting leak K current (I_L) (9-12). Blockade of these conductances results in depolarization of the cell, increase in input resistance, and enhancement of spontaneous activity and, perhaps, enhanced reactivity to afferent stimulation. Other reports suggest that ACh may actually facilitate a K current (I_K) (13)). In addition, the muscarinic agonist CCh is reported to modulate a calcium dependent inward current (14,15). One effect of particular interest is the ACh-induced suppression of the excitatory postsynaptic potentials (EPSP) evoked by stimulation of the Schaffer collateral/commissural fibers (15, 17). This effect is assumed to be mediated by presynaptic muscarinic receptors. We have recently provided evidence to suggest that this

inhibitory effect of CCh on synaptic potentials is likely to be mediated by an M3 receptor (18). These effects can be seen in most hippocampal neurons with relatively high concentrations of CCh, and are blocked by atropine. The receptor types associated with the various muscarinic effects of CCh are not entirely clear; the blockade of I_M and I_L are probably mediated by a muscarinic M1 receptor, and the blockade of I_{AHP} by an M2 receptor (11, 12), but the lack of highly specific ligands for most of the muscarinic receptors hampers further progress in this field.

ACh activates second messenger systems involving phospholipases, protein kinase C, and inositol trisphosphate (IP_3) (19) as well as blockade of production of cAMP by M2, M4 receptors (20). These, in turn, may release calcium from internal stores which can act on a number of secondary cellular processes. ACh can also cause a rise of intracellular calcium ($[Ca]_i$) simply by closing potassium channels, allowing the membrane to depolarize and activate voltage-gated calcium currents (21). These actions will trigger several postsynaptic calcium-related, second messenger processes.

We have recently described a fast onset, selective potentiation of reactivity of hippocampal neurons to application of the glutamate agonist N-methyl D-aspartate (NMDA). This was the first demonstration of an interaction between a slow and a fast neurotransmitter, at the second messenger level in the hippocampus. The cholinergic potentiation of NMDA responses is assumed to involve activation of the IP_3 cascade (22,23), and is mediated by an M2 receptor. It may function to enhance the NMDA component of the EPSP, and allow more calcium to flow into the cell, and initiate calcium dependent plasticity. This effect is short lasting, and a recovery of NMDA responses was seen immediately following removal of the cholinergic stimulation(22).

The fact that ACh can have different actions on different cell types in the hippocampus (24) complicates the already complex issue of which action of acetylcholine is most relevant to neuronal plasticity. Thus, M2 receptors are found on pyramidal neurons, but are also in high concentrations on interneurons located in stratum oriens (25). Likewise, all other muscarinic receptors may have a selective preferential localization or action on different cell types and their final effects on hippocampal circuits will be a complex function of their activity and strategic location in the affected cells in the circuit.

Acetylcholine and Neuronal Plasticity

Insight into the role of ACh in the cellular and molecular aspects of plasticity has been gained by examining the effects of ACh on LTP. In the dentate gyrus of rat hippocampus, muscarinic activation facilitates LTP induction (26) and physostigmine, an inhibitor of AChE, causes potentiation of population spikes resembling LTP (27,28). The situation becomes more complex, as in area CA3-mossy fiber system, low concentrations of CCh suppress tetanus-induced LTP (29) via activation of a high affinity M2 receptor (note, however that Williams and Johnston, (30) obtained similar results with high concentration of CCh). In these same neurons, activation of M1 receptors facilitates LTP induction. This effect is opposite to that seen in the dentate gyrus and area CA1 (below), probably related to the fact that LTP in the mossy fiber system is mediated by different mechanisms from those in the other areas, most likely via presynaptic modulation of transmitter release.

In area CA1, CCh can enhance LTP (31) and the muscarinic antagonist atropine can suppress associative LTP (32). Cholinergically induced rhythmic activity, obtained with higher concentrations of CCh, can enhance plasticity of neurons in response to afferent stimulation (33). It has also been suggested that anticholinergic drugs suppress the ability of area CA1 to express LTP (34).

We have recently found that bath application of low concentrations (0.2-0.5 μ M) of CCh induces long term potentiation (LTP) of reactivity to afferent stimulation in the hippocampus ((35), Fig. 1) This muscarinic LTP (LTPm) sharply contrasts with the response to a higher concentration of CCh, resulting in inhibition of the EPSP (Fig. 1). In fact it looks as if the two effects of CCh are mediated by totally different mechanisms. LTPm depends on cholinergic stimulation for its initiation but not for its maintenance, as it is sustained long after CCh is removed from the medium(35). LTPm is independent of activation of an NMDA receptor, as it can be produced in the presence of the NMDA antagonist 2-APV. However, it shares similarities with tetanic LTP which lie downstream of the involvement of the NMDA receptor in LTP induction e.g. application of an even lower CCh concentration (0.1 μ M CCh), while having no observable effect of its own, reduces the threshold for tetanic LTP induction. Furthermore, saturation of the tetanic LTP mechanism occludes the ability of CCh to produce LTPm, and vice versa (Fig. 2), indicating that the two types of stimulation share the same downstream mechanism.

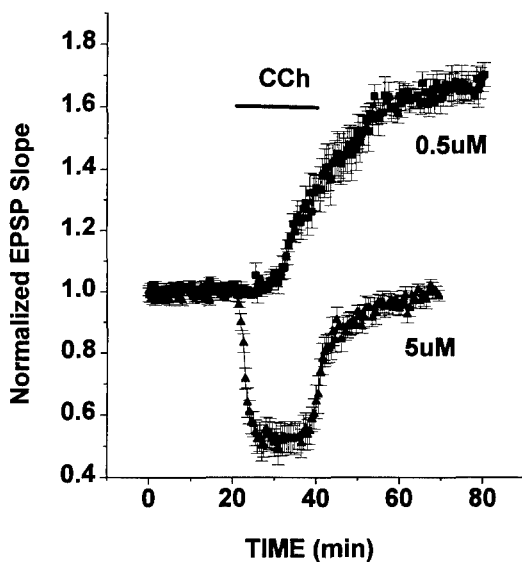


Fig. 1:

Dose dependent effects of CCh on reactivity of hippocampal CA1 cells to afferent stimulation.

Extracellularly recorded population EPSP slopes are plotted before, during and after perfusion of the slice with either 0.5 μ M or 5 μ M CCh. The lower concentration of CCh produces a slow onset, long lasting enhancement of population EPSP slope, whereas the higher concentration of CCh produces a fast onset, fast recovery of depression of the EPSP. Ordinate EPSP slope, relative to control, predrug level. Abscissa, time. (Modified from 18)

LTPm is likely to be mediated by a genuine long term change in postsynaptic reactivity to activation of the AMPA receptor, and not by a presynaptic change in fiber excitability or release properties, as indicated by the responses to paired pulse stimulation and by the lack of change in presynaptic volley (18). Moreover, cells in the hippocampus expressed a prolonged enhancement of their reactivity to AMPA following a exposure to CCh, whereas they did enhance their

reactivity to NMDA only transiently, as seen before (18, 22, 23). This indicates that CCh may exert its action through some interaction with a second messenger system, which modulates the AMPA receptor. Interestingly, this second messenger interaction takes a fairly long time to develop, unlike the interaction with the NMDA receptor. In an attempt to begin deciphering this second messenger system, we first found that LTPm is dependent on a rise of intracellular calcium, but not on an influx of calcium into the cell during exposure to the drug (35). Next we found that LTPm is likely to involve protein phosphorylation as it is blocked by antagonists of both a serine/threonine kinase (H7), (35) and a tyrosine kinase (Auerbach and Segal, unpublished observations). Also, we found that this effect of CCh is temperature dependent, and is not produced at room temperature (Fig 3). We now pursue the analysis of the second messenger(s) that may be activated by CCh to enhance reactivity to AMPA in these hippocampal neurons.

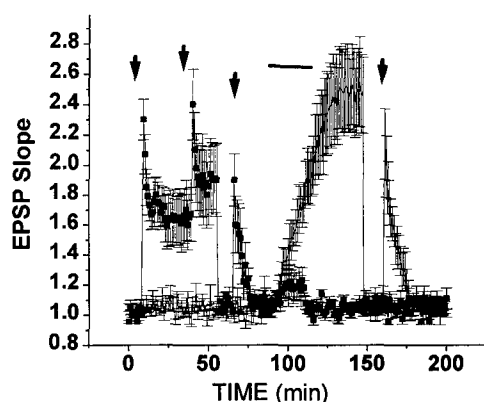


Fig. 2:

LTPm and tetanus-induced LTP share a common effector. population spike data recorded from one site, in response to stimulation of two inputs. The mechanism of tetanic LTP was first saturated in input 1, the magnitude of stimulation was then reduced to verify that the response is not at the saturation level, and a third tetanic stimulation already produced only short term potentiation. Addition of CCh ($0.75\mu\text{M}$, bar) had no effect on the response to stimulation of input 1, but produced the typical potentiation of the response to input 2. Once LTPm was established, suprathreshold tetanus

to input 2 failed to further enhance already potentiated response. Arrows indicate tetanic stimulations (100 pulses, 100Hz) (modified from 35).

The pharmacology of LTPm was studied alongside the inhibition of EPSP produced by the higher concentration of CCh (18). The latter effect is mediated by an M3 receptor, as it was blocked by the M3 antagonist 4-DAMP (36). LTPm was unaffected by M1 agonists or antagonists, but was blocked by methoctramine or AFDX-116, both M2 antagonists (8, 37).

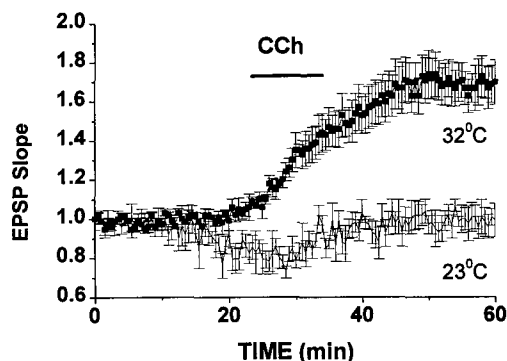


Fig 3:

Temperature dependence of LTPm. Slices were maintained in either $23-24^{\circ}\text{C}$ ($n=6$ slices) or in 32°C ($n=5$) and superfused with $0.5\mu\text{M}$ CCh. Only the slices maintained at the higher temperature expressed the robust LTPm.

Interestingly, once the inhibition produced by higher concentration of CCh was blocked by 4-DAMP, no LTPm surfaced. If indeed LTPm is activated by an high affinity M2 receptor, why should it not be activated when the inhibitory effect, mediated by a lower affinity M3 receptor is blocked? These results indicate that there may be another player in this interaction of CCh with hippocampal neurons in the slice. One important candidate for such an interaction are the interneurons of the hippocampus, particularly those residing in stratum oriens of CA1 (24). These have been shown to be innervated by cholinergic fibers of septal origin, and to possess a variety of muscarinic receptors. A detailed analysis of the interneuronal interactions affected by CCh is underway, using paired recordings of interneurons/pyramidal neurons and the inhibitory synaptic potential connecting them. An encouraging result was obtained thus far; blockade of GABAergic inhibition uncovered LTPm in response to high concentration of CCh (18). It appears that high CCh concentration (5 μ M) may have activated an interneuron, which shunts the ability of the pyramidal neurons to express LTPm. This hypothesis will be tested more directly in future experiments.

The long lasting change in efficacy of transmission in the schaffer collateral/commissural input to CA1 pyramidal neurons, produced by exposure to low concentration of CCh, is, by and large, the closest association of ACh with neuronal plasticity in the brain, i.e. LTPm is long lasting, as is tetanic LTP and in fact, it shares downstream mechanisms with tetanic LTP. As mentioned above, even lower concentrations of CCh can facilitate tetanic LTP, and thus, a coincidence between a burst of afferent discharge and a cholinergic input may facilitate reactivity of the hippocampus to afferent stimulation. Another indication that LTPm might be related to brain cholinergic association with learning and memory comes from our observation (Auerbach and Segal, unpublished observations) that slices taken from aged rats lack LTPm. By comparison with other reported cholinergic deficits (2), this one is very large indeed, and may underly the inability of aged rats to learn spatial cognitive tasks.

Having to activate a nearby, high affinity M2 receptor, is clearly a more realistic and easier task for acetylcholine released from terminals in stratum oriens, than having to bind to a remote, low affinity M1 receptor residing on apical dendrites, which may affect excitability of the cell by blocking K channels. While the second messenger systems activated by the M2 receptor to cause a rise in the AMPA receptor sensitivity to glutamate, and the specific cell types involved in this action are still not entirely clear, the gap between the cholinergic involvement in memory, and the physiological action of ACh at the single cell is beginning to be bridged.

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REGULATION OF PHOSPHOLIPASE C AND D ACTIVITIES BY SMALL MOLECULAR WEIGHT G PROTEINS AND MUSCARINIC RECEPTORS

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Summary

The role of small molecular weight guanine nucleotide-binding proteins (G proteins) of the Rho family in muscarinic acetylcholine receptor (mAChR) signaling to phospholipase C (PLC) and phospholipase D (PLD) was studied in human embryonic kidney (HEK) cells, stably expressing the human m3 receptor subtype. Evidence for the involvement of Rho proteins in m3 mAChR signaling to both phospholipases is based on findings obtained with *Clostridium (C.) difficile* toxin B and *C. botulinum* C3 exoenzyme, both of which specifically, although by different mechanisms, inactivate Rho family G proteins. Toxin B potently inhibited both the mAChR-stimulated PLC and PLD activities in intact cells as well as the stimulation of both phospholipases by the stable GTP analog GTP γ S in permeabilized cells, the latter effect being mimicked by C3 exoenzyme. In contrast, PLC and PLD activities, measured in the presence of exogenous phosphatidylinositol 4,5-bisphosphate [PtdIns(4,5)P₂], a substrate and cofactor for PLC and PLD, respectively, were not altered. These data suggested that the Rho-inactivating toxins inhibit stimulation of PLC and PLD by reducing the cellular level of PtdIns(4,5)P₂, which was indeed found with both toxin B and C3 exoenzyme. In agreement with a crucial role of cellular PtdIns(4,5)P₂ supply for PLC signaling, we observed that short-term agonist (carbachol) treatment of HEK cells caused a long-lasting increase in PtdIns(4,5)P₂ level, accompanied by a potentiation of receptor- and G protein-stimulated inositol phosphate formation. Finally, studies with tyrosine kinase and tyrosine phosphatase inhibitors strongly suggest that PtdIns(4,5)P₂ synthesis and mAChR-stimulated PLD activity in HEK cells apparently also involve a tyrosine phosphorylation-dependent mechanism(s). Thus, m3 mAChR signaling to PLC and PLD in HEK cells requires the concerted action of various intracellular components, most notably the complex regulation of PtdIns(4,5)P₂ synthesis.

Key Words: phospholipase C, phospholipase D, RhoA, m3 AchR signaling

Activation of phospholipase C (PLC) enzymes, leading to the generation of the two second messengers, inositol 1,4,5-trisphosphate (InsP₃) and diacylglycerol, from phosphatidylinositol 4,5-bisphosphate [(PtdIns(4,5)P₂), is a ubiquitous response of eukaryotic cells downstream of various tyrosine kinase receptors and heptahelical receptors coupled to heterotrimeric guanine nucleotide-binding proteins (G proteins) (1). G protein-coupled receptors activate PLC isoforms of the β family by two distinct mechanisms, either involving GTP-liganded α subunits of the pertussis toxin (PTX)-insensitive G_q class of G proteins or free $\beta\gamma$ dimers of the PTX-sensitive G_i type G

proteins (1,2). As shown by reconstitution studies, apparently only three components are required for PLC activation by G protein-coupled receptors, *i.e.*, the receptor, a PLC- β isozyme, and the α subunit or the free $\beta\gamma$ dimer of the heterotrimeric G protein (1). However, in intact cells, the regulatory mechanisms involved in PLC stimulation are far more complex. Particular the supply of the PLC substrate, PtdIns(4,5) P_2 , has a great impact on the appropriate signaling of various receptors to PLC (3,4).

Activation of phospholipase D (PLD) by G protein-coupled receptors has been reported in a wide range of cell types in response to various hormones and neurotransmitters (5,6). The type of heterotrimeric G protein, PTX-sensitive or -insensitive, involved in receptor signaling to PLD differs from one cell and receptor type to another. Besides the receptors themselves, various intracellular components such as protein kinase C, tyrosine kinases, and small molecular weight G proteins, particularly ARF and Rho family G proteins, have been implicated as regulators of PLD activity (5,6). In addition, PtdIns(4,5) P_2 has recently been identified as an essential regulatory component of PLD activity (7). However, the exact mechanisms linking cell surface receptors to PLD are unknown.

We have studied receptor signaling to PLC and PLD in human embryonic kidney (HEK) cells, stably expressing the human m3 muscarinic acetylcholine receptor (mAChR) subtype. The receptor couples to $G_{q/11}$ proteins and causes efficient stimulation of both phospholipases in a PTX-insensitive manner (8). Stimulation of PLC and PLD by the m3 mAChR is apparently mediated by quite distinct mechanisms. For example, short-term agonist (carbachol) treatment of HEK cells causes a rapid and long-lasting desensitization of PLD stimulation, whereas mAChR-induced PLC activation is potentiated (9). Furthermore, PLC-derived cellular events, including protein kinase C activation and a rise in intracellular calcium, are apparently not involved in mAChR stimulation of PLD in HEK cells (8). Previously, we reported that a tyrosine phosphorylation-dependent mechanism(s) and ARF proteins participate in m3 mAChR stimulation of PLD activity in HEK cells (8,10). Recent studies with the Rho-inactivating agents, toxin B from *Clostridium (C.) difficile* and C3 exoenzyme from *C. botulinum*, then indicated that mAChR signaling to both PLC and PLD in HEK cells is dependent on Rho proteins (11,12). Therefore, in the present study, the regulation of the levels of PtdIns(4,5) P_2 , which acts as the substrate and cofactor for PLC and PLD, respectively, and concomitant alterations in PLC and PLD activities were examined. We report here that PtdIns(4,5) P_2 synthesis in HEK cells is apparently controlled by various mechanisms, including Rho proteins, tyrosine phosphorylation and the m3 mAChR, and that alterations in PtdIns(4,5) P_2 levels may, at least partially, explain the concomitant changes in PLC and PLD activities.

Methods

Cell culture. The culture conditions of HEK cells stably expressing the human m3 mAChR were as reported previously (8,9). For experiments, cells subcultured in Dulbecco's modified Eagle's F-12 medium with 10 % fetal calf serum were grown to near confluence.

Assays of PLC and PLD activities in intact and permeabilized HEK cells. For measurement of HEK cell PLC and PLD activities, cellular phospholipids were labeled by incubating monolayers of cells for 20-24 h with *myo*-[3H]inositol and/or [3H]oleic acid in growth medium (8,9). Toxin B treatment of HEK cells and measurements of PLC and PLD activities in intact and digitonin-permeabilized cells were performed as described in detail before (9,11,12). HEK cell treatment with carbachol, followed by agonist wash-out and assay of PLC activity 30 min later, was performed as described before (9).

Assay of PLD activity in HEK cell membranes. PLD activity of HEK cell membranes was measured in the presence of detergent as described in detail before (13). The assay was performed for 60 min at 30°C in an incubation medium containing 200 μ M [3 H]phosphatidylcholine (PtdCho) (about 500,000 cpm/assay), 50 mM HEPES, pH 7.0, 1 mM EGTA, 1 mM sodium oleate, 1 mM sodium cholate, 2 % ethanol, and 200 μ g membrane protein. Formation of [3 H]phosphatidylethanol (PtdEtOH) was analyzed as described before (8,9).

Phosphoinositide analysis. Cellular phosphoinositides were labeled by incubating nearly confluent monolayers of HEK cells with *myo*-[3 H]inositol for 20-24 h in growth medium, followed by treatment of intact cells for 24 h without and with toxin B (50 pg/ml) or of digitonin-permeabilized cells for 60 min without and with C3 exoenzyme (12 μ g/ml) in the presence of 50 μ M NAD (12). mAChR effects on the phosphoinositide metabolism were studied as follows: HEK cell monolayers labeled with *myo*-[3 H]inositol were treated for 2 min with and without 1 mM carbachol, followed by agonist washout and 10 min treatment with 10 mM LiCl 30 min later (14). The phosphoinositides were extracted and analyzed as described in detail before (12,14).

Data presentation. Data shown are mean \pm SD (n=3) from one experiment, repeated at least twice.

Results

m3 mAChR and Rho regulation of PLC activity and PtdIns(4,5)P₂ level. We have previously shown that treatment of m3 mAChR-expressing HEK cells with *C. difficile* toxin B, which glucosylates and thereby inactivates Rho family G proteins (15), causes a concentration- and time-dependent decrease in receptor-stimulated inositol phosphate formation (12). In addition, PLC stimulation by the direct G protein activators, AlF₄⁻ and guanosine 5'-[γ -thio]triphosphate (GTP γ S), studied in intact and permeabilized cells, respectively, was also inhibited by treatment with toxin B, whereas basal PLC activity was not affected. C3 exoenzyme, which ADP-ribosylates Rho proteins (16), mimicked the inhibitory effect of toxin B on GTP γ S-stimulated inositol phosphate formation (Fig. 1, left panel). Finally, toxin B and C3 exoenzyme also reduced the carbachol- and GTP γ S-stimulated production of InsP₃ in intact and digitonin-permeabilized HEK cells, respectively (12). Treatment of HEK cells with toxin B induced rounding-up of the cells (11), indicating disruption of the actin cytoskeleton. However, two other agents, namely cytochalasin B and *C. botulinum* C2 toxin, which mimicked the effect of toxin B on the HEK cell morphology but without involving Rho proteins (17), had no effect on mAChR-stimulated PLC activity (12). Additionally, toxin B treatment of HEK cells did not cause mAChR internalization or down-regulation and also did not interfere with receptor coupling to heterotrimeric G proteins (11).

Studies in mouse fibroblasts (18) and human platelets (19) provided evidence that members of the Rho protein family are involved in the synthesis of the PLC substrate, PtdIns(4,5)P₂. Therefore, to elucidate the role of Rho proteins in receptor signaling to PLC, we performed phosphoinositide analysis in HEK cells. Toxin B and C3 exoenzyme reduced the level of PtdIns(4,5)P₂ by about 40-50 % in intact and digitonin-permeabilized cells, respectively (Fig. 1, right panel), whereas the levels of phosphatidylinositol (PtdIns) and phosphatidylinositol 4-phosphate (PtdIns4P) were not affected (12). Accordingly, when PLC activity was measured with exogenous PtdIns(4,5)P₂ as enzyme substrate, GTP γ S- as well as AlF₄⁻-stimulated activities were not altered by prior toxin B treatment (12). These data suggested that toxin B and C3 exoenzyme, both inactivating Rho proteins, inhibit m3 mAChR signaling to PLC in HEK cells most likely by reducing the cellular substrate supply.

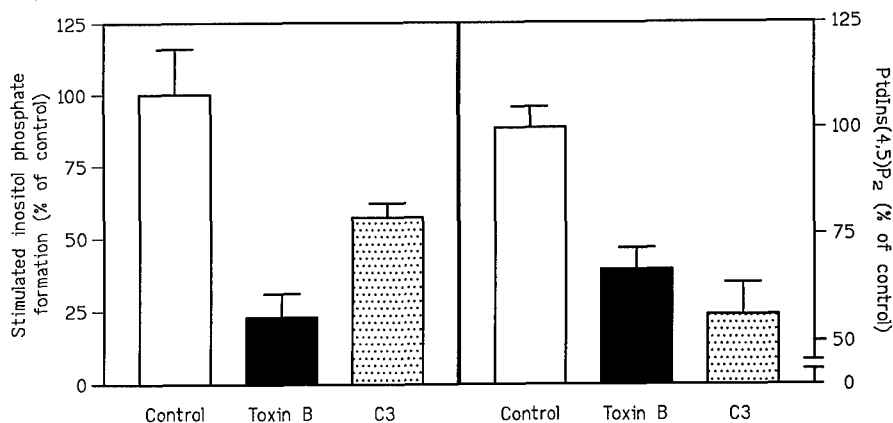


Fig. 1.

Effects of toxin B and C3 exoenzyme on the stimulus-induced inositol phosphate formation and the cellular level of PtdIns(4,5)P₂ in m3 mAChR-expressing HEK cells. Left panel: The effect of toxin B treatment (50 pg/ml, 24 h) on [³H]inositol phosphate production stimulated by carbachol (1 mM) was determined in intact HEK cells, while the influence of C3 exoenzyme (12 μg/ml) was studied on GTPγS (100 μM)-stimulated [³H]inositol phosphate formation in digitonin-permeabilized cells. Stimulus-induced [³H]inositol phosphate formation is given as % of controls in untreated cells. Right panel: The level of [³H]PtdIns(4,5)P₂ was quantified in HEK cells labeled with *myo*-[³H]inositol and subsequently treated without and with 50 pg/ml toxin B (intact cells) or 12 μg/ml C3 exoenzyme (permeabilized cells). Data are given as % of untreated cells.

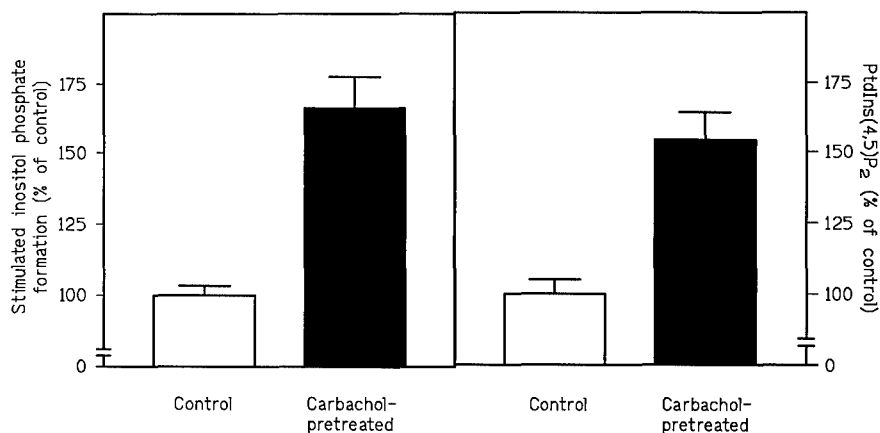


Fig. 2.

Effects of carbachol pretreatment on the carbachol-stimulated inositol phosphate formation and the cellular level of PtdIns(4,5)P₂ in m3 mAChR-expressing HEK cells. Left panel: Carbachol (1 mM)-stimulated [³H]inositol phosphate formation was measured in HEK cells pretreated for 2 min without and with 1 mM carbachol. Stimulated product formation is given as % of untreated control cells. Right panel: [³H]PtdIns(4,5)P₂ was quantified in HEK cells pretreated for 2 min without and with 1 mM carbachol. Data are given as % of untreated control cells.

In previous studies we demonstrated that agonist (carbachol) pretreatment of m3 mAChR-expressing HEK cells differentially affects receptor signaling to PLC and PLD (9). Whereas short-term (2 min) carbachol treatment abrogated receptor-stimulated PLD activity, receptor stimulation of PLC activity was sensitized. As exemplified in Fig. 2 (left panel), m3 mAChR-mediated PLC stimulation, measured as accumulation of total inositol phosphates, was enhanced markedly (by about 70 %) in cells pretreated for 2 min with carbachol. Furthermore, carbachol pretreatment also markedly enhanced, by 2- to 3-fold, stimulation of PLC activity by the endogenously expressed purinergic and thrombin receptors and upon direct activation of G proteins by AlF_4^- and $\text{GTP}\gamma\text{S}$ (14). Previous studies performed in human neutrophils and rat pancreatic acinar cells indicated that $\text{PtdIns}(4,5)\text{P}_2$ synthesis can be stimulated by G protein-coupled receptors (20,21). As the data obtained with the Rho-inactivating toxins suggested that the supply of $\text{PtdIns}(4,5)\text{P}_2$ has a crucial impact on receptor signaling to PLC in HEK cells as well, the level of phosphoinositides was analyzed in carbachol-pretreated cells. As illustrated in Fig. 2 (right panel), such treatment raised the level of $\text{PtdIns}(4,5)\text{P}_2$ by about 50 % above control, whereas the level of PtdIns was not altered and that of $\text{PtdIns}4\text{P}$ reduced by about 40 % (14). In agreement with a major role of the rise in $\text{PtdIns}(4,5)\text{P}_2$ level in potentiation of PLC signaling, no alterations in PLC activities were observed when measured with exogenous $\text{PtdIns}(4,5)\text{P}_2$ as enzyme substrate (14).

Role of $\text{PtdIns}(4,5)\text{P}_2$ in regulation of PLD activity. Members of the Rho protein family have been reported to stimulate PLD in various *in vitro* preparations (22-24). To study the involvement of Rho proteins in receptor signaling to PLD, we examined the effect of toxin B on mAChR-mediated stimulation of PLD in HEK cells stably expressing the m3 subtype (11). Toxin B treatment of the cells potently and efficiently inhibited mAChR-stimulated PLD activity. For example, upon treatment of HEK cells with 50 pg/ml toxin B for 24 h, the carbachol-evoked PLD stimulation was reduced by about 80 % (11). Toxin B treatment also inhibited PLD stimulation by direct activation of heterotrimeric G proteins with AlF_4^- and by activation of the small G protein ARF with $\text{GTP}\gamma\text{S}$, measured in intact and permeabilized cells, respectively. The latter inhibition was mimicked by C3 exoenzyme, implicating the involvement of Rho proteins in both receptor- and G protein-mediated PLD stimulation (11). Basal PLD activities, measured in intact or permeabilized HEK cells, were not affected by treatment with toxin B or C3 exoenzyme. Furthermore, treatment of HEK cells with cytochalasin B or *C. botulinum* C2 toxin, both mimicking the cell morphology alterations induced by toxin B (see above), did not affect mAChR-mediated PLD stimulation (11). As the Rho-inactivating agents reduced the cellular level of $\text{PtdIns}(4,5)\text{P}_2$ in HEK cells (see above) and as this polyphosphoinositide has been reported to serve as an essential cofactor for PLD activity (7,25), it was tempting to speculate that inhibition of PLD stimulation by the two toxins was caused by depletion of $\text{PtdIns}(4,5)\text{P}_2$. Therefore, we studied first whether $\text{PtdIns}(4,5)\text{P}_2$ is a cofactor for PLD activity in HEK cells as well and secondly whether PLD activity, reduced by toxin B treatment, could be restored by the addition of $\text{PtdIns}(4,5)\text{P}_2$ when measured with exogenous PtdCho vesicles as substrate. As shown in Fig. 3, PLD activity in membrane preparations of toxin B-treated HEK cells was reduced by about 50 % compared to control membranes. Addition of $\text{PtdIns}(4,5)\text{P}_2$, but not $\text{PtdIns}4\text{P}$, PtdIns and various other phospholipids (13), specifically increased PLD activity. Most important, in the presence of $\text{PtdIns}(4,5)\text{P}_2$ the inhibitory effect of toxin B treatment on basal and $\text{GTP}\gamma\text{S}$ -stimulated PLD activities was fully reversed (13).

mAChR stimulation of PLD in HEK cells apparently involves a tyrosine kinase-dependent mechanism (8). First, treatment of HEK cells with pervanadate, an inhibitor of protein tyrosine phosphatases, increased PLD activity. Second, and as exemplified in Fig. 4 (left panel), pretreatment of HEK cells with the tyrosine kinase inhibitor, genistein, nearly completely abolished carbachol-induced PLD stimulation (8). Genistein (and pervanadate), however, did not

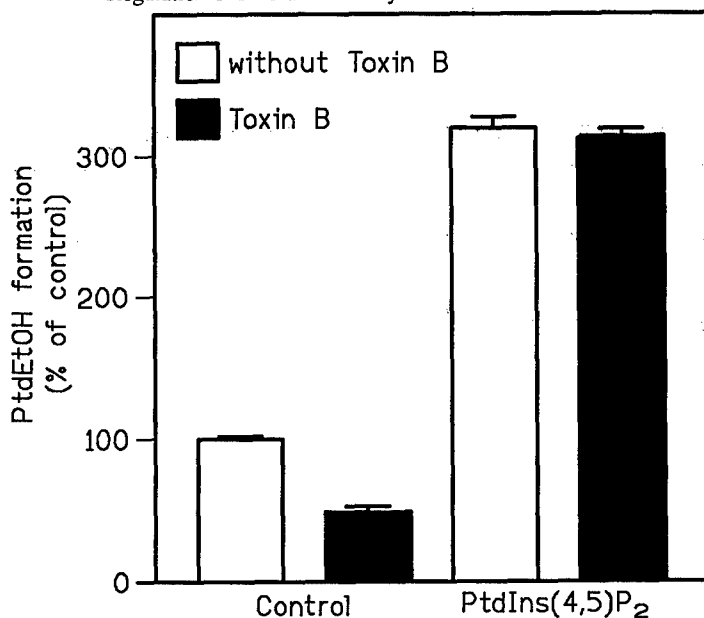


Fig. 3.

Effects of PtdIns(4,5)P₂ on PLD activities in control and toxin B-treated HEK cell membranes. HEK cells were treated for 24 h without and with 50 pg/ml toxin B, followed by membrane preparation and measurement of PLD activities with exogenous substrate ([³H]PtdCho) in the absence (Control) and presence of 25 μM PtdIns(4,5)P₂. Formation of [³H]PtdEtOH is given as % of that in control membranes measured in the absence of PtdIns(4,5)P₂.

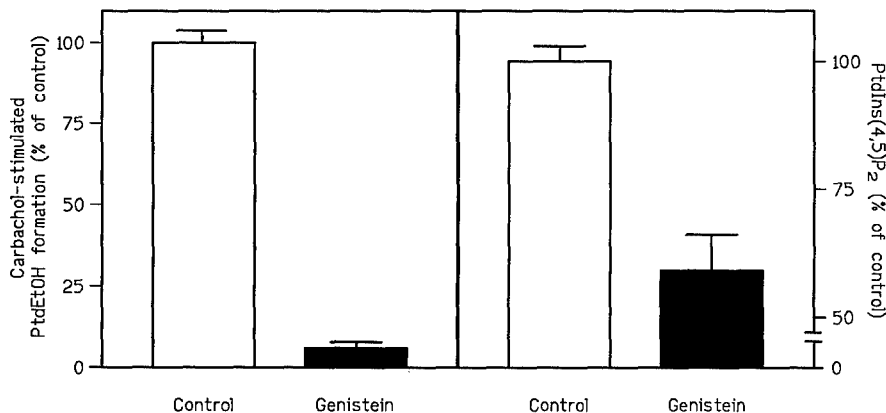


Fig 4.

Effects of genistein on carbachol-induced PLD stimulation and the cellular level of PtdIns(4,5)P₂ in m3 mAChR-expressing HEK cells. Left panel: HEK cells labeled with [³H]oleic acid were pretreated or not with 100 μM genistein for 30 min, followed by measurement of carbachol (1 mM)-stimulated [³H]PtdEtOH production. Stimulated [³H]PtdEtOH formation is given as % of control in untreated cells. Right panel: The level of [³H]PtdIns(4,5)P₂ was quantified in *myo*-[³H]inositol-prelabeled HEK cells treated for 60 min with and without 100 μM genistein. Data are given as % of control in untreated cells.

affect the carbachol-induced ARF translocation from the cytosol to HEK cell membranes (data not shown), which is apparently a sign and prerequisite for PLD activation by the m3 mAChR (10). Therefore, we examined whether the tyrosine kinase inhibitor genistein potentially inhibits receptor-mediated PLD stimulation by a likewise reduction of the cellular PtdIns(4,5)P₂ level, as toxin B and C3 exoenzyme did. As shown in Fig. 4 (right panel), the level of PtdIns(4,5)P₂ was markedly reduced by genistein (100 μ M) treatment and amounted to only 50 % of that in untreated control HEK cells, whereas the levels of PtdIns and PtdIns4P were not altered compared to untreated control cells. These results indicated that a genistein-sensitive tyrosine kinase is apparently involved in the regulation of PtdIns(4,5)P₂ synthesis in HEK cells. Corroborating this hypothesis, pervanadate, which increased HEK cell PLD activity, was found to markedly increase (by at least 2-fold) the level of PtdIns(4,5)P₂ in HEK cells, while the level of PtdIns was not altered and that of PtdIns4P increased by only about 50 % (data not shown).

Discussion

Toxin B and C3 exoenzyme modify and thereby inactivate members of the Rho protein family by highly specific covalent modifications (15,16). Toxin B monoglucosylates RhoA, Rac1, and Cdc42, while C3 exoenzyme ADP-ribosylates RhoA, B, and C. The combined analysis with both tools working on distinct members of the Rho protein family allows us to conclude that toxin B and C3 exoenzyme evoke their inhibitory effects on m3 mAChR signaling in HEK cells most likely by an action on RhoA proteins (11,12). Our data furthermore suggest that Rho proteins and their cellular targets are involved in mAChR stimulation of both PLC and PLD. Rho proteins have recently been reported to stimulate PtdIns4P 5-kinase activity (18), the enzyme finally responsible for PtdIns(4,5)P₂ synthesis. This phosphoinositide not only serves as PLC substrate, but PtdIns(4,5)P₂ also acts as an important cofactor for PLD activity (7,25) and additionally stimulates guanine nucleotide exchange on ARF, the other small molecular weight G protein apparently involved in mAChR stimulation of PLD activity (10). Therefore, we hypothesized that Rho proteins may regulate both signaling pathways by controlling the cellular level of PtdIns(4,5)P₂. Indeed, phosphoinositide analysis in toxin B- and C3 exoenzyme-treated HEK cells revealed that treatment with either Rho-inactivating toxin caused a significant reduction in the level of PtdIns(4,5)P₂, without altering those of PtdIns and PtdIns4P. Most important, when PLC activities were measured with exogenous PtdIns(4,5)P₂ as enzyme substrate, stimulated PLC activities were not altered by prior toxin B treatment. Furthermore, addition of PtdIns(4,5)P₂ fully restored basal and GTP γ S-stimulated PLD activities in membranes of toxin B-treated HEK cells. These results indicate that toxin B and C3 exoenzyme do not modify PLC and PLD enzymes *per se* and, furthermore, suggest that Rho proteins may not directly stimulate PLD activity in HEK cells. Since alternative mechanisms for PLD activation by Rho proteins in other cellular systems have been proposed (22-24), final proof for this hypothesis will require reconstitution of Rho proteins with a purified PLD enzyme. The first human PLD has recently been cloned (26).

Synthesis of PtdIns(4,5)P₂ in HEK cells is apparently regulated by a concerted action of various signal transduction components. Besides Rho proteins, a yet unidentified tyrosine kinase seems to play a crucial role in the regulation of PtdIns(4,5)P₂ synthesis, as demonstrated by studies with tyrosine kinase and tyrosine phosphatase inhibitors. Moreover, synthesis of PtdIns(4,5)P₂ seems to be under control by the m3 mAChR, suggesting a complex receptor regulation of phosphoinositide metabolism, including degradation and synthesis. Future experiments will have to elucidate the exact coupling mechanisms, by which the m3 mAChR signals to the various signal transduction components, *e.g.*, ARF, Rho, PtdIns4P 5-kinase, and the putative tyrosine kinase(s), and thereby stimulates PLC and PLD activities.

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REGULATION OF MUSCARINIC RECEPTOR EXPRESSION AND FUNCTION IN CULTURED CELLS AND IN KNOCK-OUT MICE

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Summary

We have investigated the molecular and cellular basis for the regulation of expression and function of the muscarinic acetylcholine receptors. Treatment of cultured chick cardiac cells with the agonist carbachol results in decreased levels of mRNA encoding the m2 and m4 receptors. Treatment of chick embryos *in ovo* with carbachol results in decreased levels of mRNA encoding the potassium channel subunits GIRK1 and GIRK4 as well as the m2 receptor. There are thus multiple pathways for the regulation of mAChR responsiveness by long-term agonist exposure. Immunoblot, immunoprecipitation, and solution hybridization analyses have been used to quantitate the regulation of mAChR expression in chick retina during embryonic development. The m4 receptor is the predominant subtype expressed early in development, while the expression of the m3 and m2 receptors increases later in development. A cAMP-regulated luciferase reporter gene has been used to demonstrate that the m2 and m4 receptors have distinct specificities for coupling to G-protein subtypes to mediate inhibition of adenylyl cyclase. This system has also been used to demonstrate that β -arrestin1 and β -adrenergic receptor kinase-1 act synergistically to promote receptor desensitization. We have isolated the promoter region for the chick m2 receptor gene, identified regions of the promoter required to drive high level expression in cardiac and neural cells, and have identified a region which confers sensitivity of gene expression to neurally active cytokines. Finally, in order to determine the role of individual receptor subtypes in muscarinic-mediated responses *in vivo*, we have used the method of targeted gene disruption by homologous recombination to generate mice deficient in the m1 receptor.

Key Words: muscarinic coupling, desensitization, heart, potassium channel, promoter, knock-out mice

Our laboratory has been interested in determining the mechanisms responsible for the regulation of the expression and function of the muscarinic acetylcholine receptors (mAChR) in order to define the molecular and cellular basis for the regulation of synaptic transmission. In this article we will describe studies on the transcriptional and post-translational mechanisms which regulate the expression of both the mAChR and the ion channels required for their action in cultured cells and in intact tissues, the functional specificity of mAChR-G protein coupling, and the elucidation of the function of individual mAChR subtypes in the nervous system *in vivo*.

Regulation of Cardiac mAChR Expression During Chick Embryonic Development

There are a number of interesting changes in both the expression and function of mAChR that occur in embryonic chick heart during development (1). While mammalian heart expresses

almost exclusively the m2 subtype, the embryonic chick heart expresses multiple subtypes of mAChR: the m2 (the most abundant), m4, and m3 (the least abundant) subtypes. In order to begin to explore the mechanisms responsible for the developmental regulation of mAChR, we used immunoprecipitation and immunoblot analyses with subtype-specific antibodies and solution hybridization using subtype-specific riboprobes to determine the regulation of expression of the cm2, cm3, and cm4 receptors at both the protein and mRNA levels in developing chick heart (2). The expression of the cm3 receptor decreases during development. This decreased expression correlates with the decreased coupling of mAChR to activation of the phospholipase C in the chick heart during embryonic development.

Regulation of Retinal mAChR Expression During Chick Embryonic Development

Several studies have also shown that there are developmental changes in the pattern of expression of mAChR during development of the chick retina. Studies using affinity alkylation and SDS gel electrophoresis demonstrated that early in development, the predominant species of mAChR migrated primarily as a large molecular weight species (86 kDa); the main form of mAChR shifted to a lower molecular weight species (72 kDa) during development (3). A similar shift in molecular weight occurred in dissociated retinal cultures. It has been suggested that a developmentally regulated secreted factor is responsible for the shift in mAChR species (4). We used immunoprecipitation and immunoblot analyses and solution hybridization to examine the expression of mAChR subtypes in the chick retina during development (2). Early in development, the main subtype is the cm4 receptor. The proportion of cm3 and cm2 receptors in the retina increased during development. Interestingly, the apparent molecular weight of the cm2 was significantly less than that of the cm3 and cm4 receptors. The change in molecular weight reported previously thus was due to the developmentally regulated appearance of the cm2 receptor. Preliminary results suggest that a similar increase in cm2 expression occurs in retinal cultures and that retinal cell culture conditioned medium induces the appearance of the cm2 receptor.

Regulation of Cardiac mAChR and G-protein Coupled Inwardly Rectifying Potassium Channel mRNA Levels by mAChR Activation

Incubation of cultured chick heart cells with the muscarinic agonist carbachol resulted in decreased levels of mRNA encoding both the cm2 and cm4 receptors. The rate of mRNA degradation was unchanged following carbachol treatment, suggesting that the decreases in mRNA levels result from a change in the rate of gene transcription. Activation of mAChRs in chick heart causes both inhibition of adenylyl cyclase (AC) activity and stimulation of phospholipase C (PLC) activity. A combination of biochemical and pharmacological approaches were used which allowed selective coupling to each of these second messenger pathways. These studies demonstrated that both inhibition of the AC pathway and stimulation of the PLC pathway are required for maximal decreases of cm2 and cm4 mRNA levels in response to activation of both homologous (i.e., muscarinic) and heterologous (viz., adenosine and angiotensin) receptors in these cells. The decreased levels of mAChR mRNA have important functional consequences, because it decreased the rate of reappearance of muscarinic receptors when the cells recover from agonist-induced down-regulation after removal of agonist (5,6).

Activation of muscarinic receptors in the heart leads to a decrease in the rate of contraction of the heart due in part to activation of a G-protein activated inwardly rectifying channel composed of two subunits, GIRK1 and GIRK4 (7). We have previously shown that the chick embryo is an attractive system for the study of the regulation of mAChR expression and function *in vivo*: administration of muscarinic agonists *in ovo* caused large decreases in the number of mAChR binding sites, mAChR-mediated inhibition of adenylyl cyclase, and the mAChR-mediated negative chronotropic effect (8,9). We tested if *in ovo* administration of the agonist carbachol had any effect on the expression of GIRK1 and GIRK4. Treatment with carbachol resulted in decreased levels of mRNA encoding both GIRK1 and GIRK4, as well as the m2 receptor, in the atria. This decreased level of channel mRNA was accompanied by a decrease in the level of GIRK1 immunoreactive polypeptide as detected by immunoblot analysis (10). Thus, persistent activation of mAChR results in decreased expression not only of the mAChR but the ion channel whose activity is regulated by the receptor.

Analysis of the Specificity of Functional Coupling and Regulation of mAChR Signaling Using a cAMP-regulated Reporter Gene

We have used the regulation of expression of a luciferase reporter gene under the transcriptional control of a cAMP-responsive element (CRE) to monitor mAChR-mediated changes in intracellular cAMP levels in transiently transfected JEG3 cells (11-13). In contrast to determination of cAMP levels by traditional biochemical methods, this approach allows measurement of changes in physiologically relevant levels of intracellular cAMP, does not require the addition of phosphodiesterase inhibitors, and is particularly convenient for experiments with transiently transfected cells, because it eliminates the effects of background levels of cAMP in the untransfected cells which will be present in the culture. This system allows one to determine the physiological responses resulting from an essentially unlimited combination of receptors, G-proteins, protein kinases, etc., in transiently transfected cells.

We have used this system to demonstrate that the m2 and m4 receptors have distinct specificities for coupling to G-protein subtypes to mediate inhibition of adenylyl cyclase activity. Transfection of either the human or chick m2 or m4 receptors into JEG3 cells results in little if any inhibition of either basal or forskolin-stimulated CRE-luciferase expression. Cotransfection of either $G_{i\alpha-2}$ or $G_{O\alpha}$, but not $G_{i\alpha-1}$ or $G_{i\alpha3}$, with the m4 receptor allowed robust inhibition of CRE-luciferase expression. In contrast, cotransfection of $G_{i\alpha-1}$, $G_{i\alpha-2}$, $G_{i\alpha3}$, or $G_{O\alpha}$ allowed the m2 receptor to mediate inhibition of CRE-luciferase expression (11, 13). Thus, the m2 and m4 receptors have distinct but overlapping specificities for coupling to G-protein subtypes to mediate inhibition of adenylyl cyclase (13).

We have tested the feasibility of using the CRE-luciferase system to study agonist-mediated desensitization. In cells transfected with the m2 receptor, preincubation with agonist has only minor effects on the subsequent ability of agonist incubation to inhibit forskolin-stimulated CRE-luciferase expression. Cotransfection with β -arrestin1 did not affect the functional response, while cotransfection with β -adrenergic receptor kinase-1 increased the ability of agonist pretreatment to attenuate functional responsiveness. In cells cotransfected with the m2 receptor, β -arrestin1, and β -adrenergic receptor kinase-1, preincubation with carbachol resulted in a complete loss of mAChR-mediated inhibition. Thus, β -arrestin1 and β -adrenergic receptor kinase-1 act synergistically to regulate mAChR responsiveness (14).

Isolation and Regulation of the Promoter for the Chick m2 Receptor Gene

We have isolated the promoter for the chick m2 receptor gene in order to identify the factors and mechanisms responsible for regulation of mAChR expression. The m2 receptor has a long (>8 kb) intron in the 5' untranslated region of the gene. This intron-exon boundary is over 320 basepairs from the 3'-most start site of transcription. The promoter region contains multiple transcriptional start sites in both cardiac and neuronal cells. Transfection experiments using cm2-promoter luciferase reporter genes demonstrated that a 789 basepair genomic fragment was sufficient to drive high level expression in cardiac cells, while an additional 1.2 kb of promoter sequence was required to drive maximal expression in SN56 septal-neuroblastoma cells. Treatment of SN56 cells with the cytokines leukemia inhibitory factor or ciliary neurotrophic factor increased mAChR number and increased the expression of the cm2 promoter reporter genes. The region which conferred sensitivity to cytokines was located in the same 1.2 kb region of the promoter required for high level neural expression (15).

Inactivation of mAChR Genes by Homologous Recombination

Muscarinic receptors are thought to have important roles in many higher functions of the brain, such as memory and learning, establishment of epileptic seizures, control of movement, and nociception. While pharmacological studies have attempted to determine the identities of the receptor subtype(s) which mediate these effects, these studies are hampered by the difficulties in unequivocally correlating pharmacological studies with the genetically defined receptors. A technique which allows analysis of the role of specific gene products in various biological responses is the technique of gene targeting by homologous recombination, which allows the investigator to create strains of mice homozygous for a mutation in any desired gene

(16). We are using this approach to create mouse strains deficient in individual mAChR subtypes to determine the role of the receptors in specific functions.

We have used gene targeting to produce mice deficient in the m1 receptor by deletion of the sequence encoding the amino terminal portion of the m1 receptor (17). Mice homozygous for the mutant allele are viable and do not produce detectable m1 receptor. These mice should be valuable in determining the roles of the m1 receptors in muscarinic-mediated responses in the central and peripheral nervous systems.

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THE M5 (m5) RECEPTOR SUBTYPE: FACT OR FICTION?

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Summary

By comparison to the other subtypes of muscarinic receptors, very little is known about the binding properties, locations, mechanisms and physiological functions of the M5 (m5)* receptor subtype. Studies of the m5 receptor have been hampered by the lack of m5-selective ligands or antibodies and a source that endogenously expresses predominantly the m5 receptor subtype. We have developed a pharmacological labeling strategy using the non-selective muscarinic antagonist [³H]NMS, in the presence of muscarinic antagonists and toxins in green mamba venom to occlude the m1-m4 receptor subtypes, to selectively label the m5 receptor subtype. This m5-selective labeling approach, along with those developed for the other four receptor subtypes, has permitted for the first time a comparison of the relative expression levels and anatomical localizations of the five muscarinic receptor subtypes in the brain. The distribution profile of the m5 receptor is distinct from the other four subtypes and is enriched in the outer layers of the cortex, specific subfields of the hippocampus, caudate putamen, olfactory tubercle and nucleus accumbens. These studies have also demonstrated that the levels of m5 receptor protein expression are apparently higher and more widespread than anticipated from previous *in situ* hybridization and immunoprecipitation studies. Taken together, the results suggest a unique and potentially physiologically important role for the m5 receptor subtype in modulating the actions of acetylcholine in the brain.

Key Words: autoradiography, muscarinic receptors, m5 receptor

Not long after the original cloning of four muscarinic receptor genes from mammalian brain, a fifth muscarinic receptor gene was identified (1,2). Nonetheless, subsequent Northern blot

*The nomenclature for muscarinic receptor subtypes has designated the big "M" terminology for the pharmacologically-defined receptors and the small "m" terminology for the molecularly-defined receptors. It is now clear that the m1 sequence corresponds to the M1 receptor, the m2 to the M2, etc. Since the receptor subtype-selectivity of the pharmacological labeling strategies described here have been validated using the individual molecular receptors expressed in transfected cells, we have utilized the small "m" terminology to refer to the respective receptors labeled by each of the subtype-selective conditions.

analyses failed to demonstrate the expression of mRNA for the m5 muscarinic receptor subtype in brain or in selected peripheral tissues under conditions that revealed the presence of the m1-m4 transcripts (1). These results suggested that the m5 receptor was expressed at very low levels, if at all, compared to the other four muscarinic receptors. *In situ* hybridization histochemical studies confirmed the relative low abundance of m5 receptor mRNA in the brain, but demonstrated a distinct localization pattern for this receptor subtype in restricted subfields of the hippocampus and basal ganglia (3,4). Within the substantia nigra pars compacta (SNc) and ventral tegmental area (VTA), m5 receptor mRNA was the only muscarinic receptor transcript co-expressed with D2 dopamine receptor mRNA (4). This discrete and highly concentrated expression of the m5 subtype suggested a unique and physiologically important role for this receptor.

The M5 Receptor: What Do We Know?

Very little is known about the pharmacology, mechanisms, locations and physiological functions of the m5 receptor compared to the other four receptor subtypes. Table 1 summarizes what we currently know about the m5 receptor. Structurally, the m5 receptor is the second largest muscarinic receptor and is most like the m3 receptor with a large i3 loop. The third cytoplasmic loop accounts for the greatest sequence diversity between receptor subtypes and also between receptors from different species. Of the five muscarinic receptors, the m5 subtype demonstrates the least homology in the i3 region between the rat and human sequences (1). Pharmacologically, no ligands have been identified with high affinity or selectivity for the m5 subtype. However, the m5 receptor demonstrates significantly lower affinities for AQ-RA 741 and AF-DX 384 compared to the m1-m4 receptors (5). Solubilization of the m5, and also the m3 receptor, is least efficient and results in large decreases in affinity of the m5 receptor for most muscarinic receptor ligands (6). Functional studies of the m5 receptor have been limited to studies in clonal cell lines over-expressing the m5 receptor protein, since no tissues or cell lines that express a predominance of the native m5 receptor have been identified. These studies have

TABLE I.

THE M5 RECEPTOR SUBTYPE: WHAT DO WE KNOW?	
STRUCTURE	<ul style="list-style-type: none"> ▶ 532 amino acids (compare to 460-480 of m1, m2 and m4) ▶ like m3 with large i3 loop ▶ least homology (80%) in i3 loop between rat and human
PHARMACOLOGY	<ul style="list-style-type: none"> ▶ no selective high affinity ligand ▶ ≥ 10-fold lower affinities than m1-m4 for AQ-RA 741 and AF-DX 384 ▶ 15-fold decrease in affinity for NMS with solubilization ▶ least efficient solubilization
MECHANISMS	<ul style="list-style-type: none"> ▶ follows subtype specific pattern for odd-numbered receptors: PLQβ via Gq, PLA$_2$, PLD, NOS, Ca$^{+2}$ influx, cAMP, reverse transformation
LOCATION	<ul style="list-style-type: none"> ▶ most restricted mRNA distribution of the five subtypes ▶ transcript expressed only in brain (also melanoma cells and microglia/macrophages) ▶ highest densities of mRNA in SNc, VTA, hippocampus, cortex and striatum ▶ co-localizes with D2 receptor in SNc

elucidated some of the biochemical signaling pathways modulated by the m5 receptor and have demonstrated that m5-mediated signal transduction follows the subtype-specific pattern for the odd-numbered muscarinic receptor subtypes. Recent studies have identified a human melanoma cell line that endogenously expresses the m5 receptor and may facilitate studies on the functional characteristics of this subtype (7).

Tools to Study Muscarinic Receptor Subtypes

Studies of the m5 receptor protein have been hampered by the lack of appropriate tools to identify, label and localize it. Pharmacological characterization and anatomical localization studies have been impossible since no ligands, toxins or antibodies are available with sufficient selectivity to specifically label the m5 receptor. Molecular probes have identified m5 receptor transcripts but not functional receptor proteins. Antibodies have been the most useful tools to study individual receptors. Receptor subtype-specific antisera have been useful for immunoprecipitation studies demonstrating the relative abundance and distribution of the five receptor proteins in brain and peripheral tissues. While these antisera have provided immunocytochemical maps of the m1-m4 receptors in the brain, similar studies of the m5 subtype have not been done due to the low sensitivity of the polyclonal antisera to this receptor and to the lack of cross-reactivity of antibodies made to portions of the human m5 sequence with the rat receptor.

Novel Pharmacological Strategies to Selectively Label Muscarinic Receptor Subtypes

Pharmacological classification of the M1-M4 receptors has for the most part relied on equilibrium binding assays with partially subtype-selective antagonists. These studies have been complicated somewhat by the overlapping affinities of most available muscarinic ligands. Thus, while these assays were the first to establish the existence of muscarinic receptor subclasses, they have not been optimal for the direct labeling and localization of the individual receptor proteins in the brain. At present, pirenzepine, a putative M1 (m1) receptor subtype-selective antagonist, remains the only radio-labeled ligand with sufficient selectivity that permits labeling of a single receptor subtype. We have recently achieved selective labeling of the m1-m4 receptor subtypes by combining the use of the distinct kinetic and equilibrium binding properties of muscarinic receptor antagonists (8,9). Selective m1 receptor labeling was achieved with low concentrations of [³H]pirenzepine as accomplished by other laboratories (8,10). Selective m2 labeling was achieved with [³H]NMS by taking advantage of 1) the subtype-selective toxins in green mamba venom to occlude the m1 and m4 subtypes (11, 12), 2) the relatively lower affinity of the m2 receptor compared to the m3 receptor for 4-DAMP (5), and 3) the rapid kinetics of antagonist binding to the m2 compared to the m5 receptor (9). Selective m3 receptor labeling with [³H]NMS was also achieved in the presence of venom to occupy the m1 and m4 receptors, AQ-RA 741 to occupy most of the m2 receptor (5, Reeve and Flynn, unpublished), and by short incubations to minimize labeling of the m5 subtype (9). Selective m4 labeling (9) was achieved with [³H]NMS in the presence of 1) guanylpirenzepine to occupy the m1 receptor, 2) AF-DX 116 to occupy the m2 receptor (13) with a subsequent dissociation to minimize [³H]NMS labeling of the m2 subtype, and 3) dextimide to occupy the m3 and m5 receptors. Each of these four labeling strategies resulted primarily in the labeling of the respective molecular receptor subtype (8,9).

Selective Labeling of the M5 Receptor Subtype

Currently, there are no subtype-selective ligands or labeling conditions available for the m5 receptor. However, the m5 receptor demonstrates at least 10-fold lower affinities for AQ-RA

741 and AF-DX 384 compared to the affinities of the m1-m4 receptor subtypes for these antagonists (5). This suggested that concentrations of these compounds that occupy most of the m1, m2, m3 and m4 receptors may leave a large fraction of the m5 receptor unoccupied and available for labeling with a non-selective tritiated ligand like NMS. In the presence of 1 μ M AQ-RA 741, 20% or less of the m1-m4 receptors were labeled with [3 H]NMS while nearly 60% of the m5 receptors were labeled. Although a significant proportion of m5 receptors were labeled compared to the other four subtypes, the higher levels of expression of the m1, m2 and m4 receptors relative to the m5 receptor in the brain, suggested that little if any m5-selective labeling would be achieved in this tissue. Thus, in order to minimize the contribution of these more abundant subtypes to the total labeling, we explored the use of venom from the green mamba, which has been shown by a number laboratories to contain muscarinic receptor subtype-selective toxins (11,12). In the presence of 30 μ g/ml crude venom, virtually none of the m1, less than 10% of the m4 receptors, and nearly all of the m5 receptors were labeled with [3 H]NMS. In the presence of both AQ-RA 741 and venom, 55% of the m5 receptors, 12% of the m3 and none of the m1, m2 and m4 receptors were labeled with [3 H]NMS (Fig. 1). This 4:1 ratio of m5:m3 labeling represented labeling primarily of the m5 subtype in the brain, since the m5 and m3 receptors are expressed at approximately equivalent levels in this tissue (14,15).

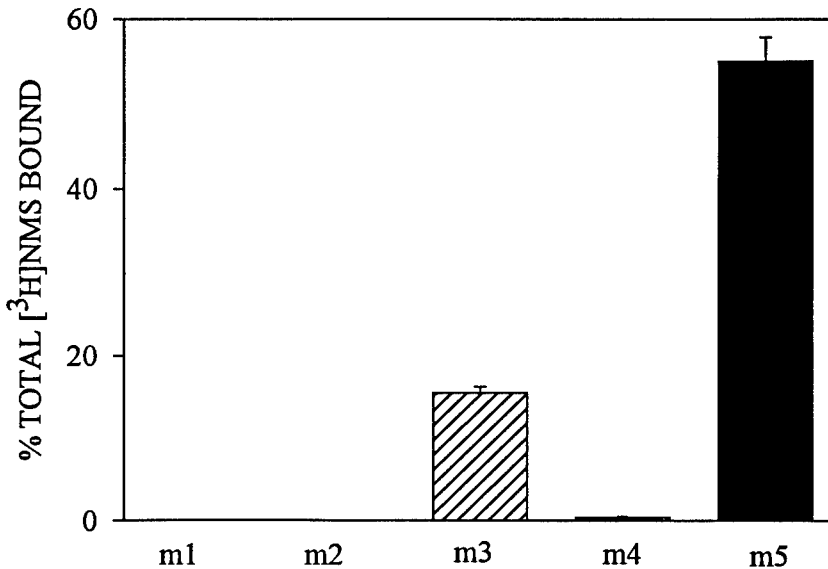


Figure 1. Proportions of m1-m5 receptors, individually expressed in CHO-K1 cells, labeled with 0.5 nM [3 H]NMS in the presence of 30 μ g/ml green mamba venom and 1 μ M AQ-RA 741. These labeling conditions resulted in nearly exclusive labeling of the m5 receptor, with a minor contribution of m3 labeling. Bars, means + standard deviation of triplicate determinations from 3 separate experiments.

Anatomical Distribution of the m5 Receptor in the Brain

Development of an m5-selective labeling strategy permitted for the first time visualization of the anatomical distribution of the m5 receptor protein. The autoradiographic [^3H]NMS labeling patterns for the total, m3 and m5 muscarinic receptor populations are shown in Figure 2. Total muscarinic receptors were visualized in the presence of 0.5 nM [^3H]NMS and m3 and m5 receptors were labeled as described above. The m5 labeling pattern was partially overlapping but distinct from the m3 pattern, as well as from the patterns previously noted for the m1-m4 receptors (8,9). The highest densities of m5 labeling were observed in the outermost layer of the cortex and the caudate putamen. Within the basal forebrain, the olfactory tubercle was the most intensely labeled region. The hippocampus showed distinct labeling of portions of the dentate gyrus and CA1 and CA2 subfields, with no labeling of the CA3 subfield. In more caudal regions, the superficial gray layer of the superior colliculus was apparent while there was an absence of labeling in the inferior colliculus. There was no distinctive m5 labeling within more posterior regions of the brain or brainstem areas. Although not visible in sagittal sections, distinct m5 labeling of the SN/VTA was apparent in coronal sections (data not shown). The SNc demonstrated the most intense labeling consistent with the noted high level of expression of m5 mRNA in this region (3,4). While there was some overlap of m3 with m5 labeling across the entire brain, there were some striking differences. Labeling of the m3 receptor was lower in the outer layer of the cortex, caudate putamen, hippocampus and olfactory tubercle compared to m5 labeling in these regions. Distinct m3 labeling was apparent in the more ventral layers of the superior colliculus, the inferior colliculus, cerebellum and specific brainstem nuclei (pontine, motor trigeminal and facial) while m5 labeling in these regions was very low or absent. These differences suggest that two distinct populations of receptors were labeled by the m3 and m5 conditions.

Physiological Role of the m5 Receptor

Since this is the first opportunity we have had to visualize the distribution of the m5 receptor protein in the brain, some speculation is warranted. The distinct m5 labeling of the outermost cortical layer is intriguing since this layer consists primarily of glial cells, apical dendrites and axons that run laterally, parallel to the pial surface. While we have previously suggested that the m5 receptor is the predominant muscarinic subtype expressed on microglia (16), it is uncertain whether this unique m5 labeling pattern results from labeling of glial cells in the superficial layers of the cortex. The m5 subtype also has been shown to be the muscarinic receptor subtype most efficiently coupled to nitric oxide synthase (NOS; 17). Taken together with the high levels of expression of the m5 receptor in the basal ganglia, a region also high in NOS expression, these findings suggest the m5 receptor may play a role in mediating NOS activation. The rather extensive distribution of the m5 subtype is surprising based on the early demonstrations by *in situ* hybridization of the relatively restricted m5 mRNA localization within the SN/VTA and its notable absence in the striatum and cortex (3,4). However, more recent studies using RT-PCR (18) have shown that the m5 subtype was uniformly expressed throughout the brain. Consistent with these studies, our autoradiograms demonstrated high levels of m5 labeling in the cortex, hippocampus, and particularly the striatum. The high level of expression of m5 receptor protein along with the co-localization of m5 receptor mRNA with dopamine D2 receptor mRNA in the SNc suggested to us that m5 receptors may be present on nigro-striatal dopaminergic terminals. We tested this hypothesis by performing 6-hydroxy-dopamine lesions of the SN/VTA in rats and examining the m5 labeling pattern in the striatum 21 days post lesion. There was nearly a complete loss of tyrosine hydroxylase staining, a marker for dopaminergic cell bodies, in the SN as well as of [^{125}I]-RTI-121 binding, a high affinity probe for the dopamine transporter, in the striatum (data not shown). These results are consistent with the degeneration

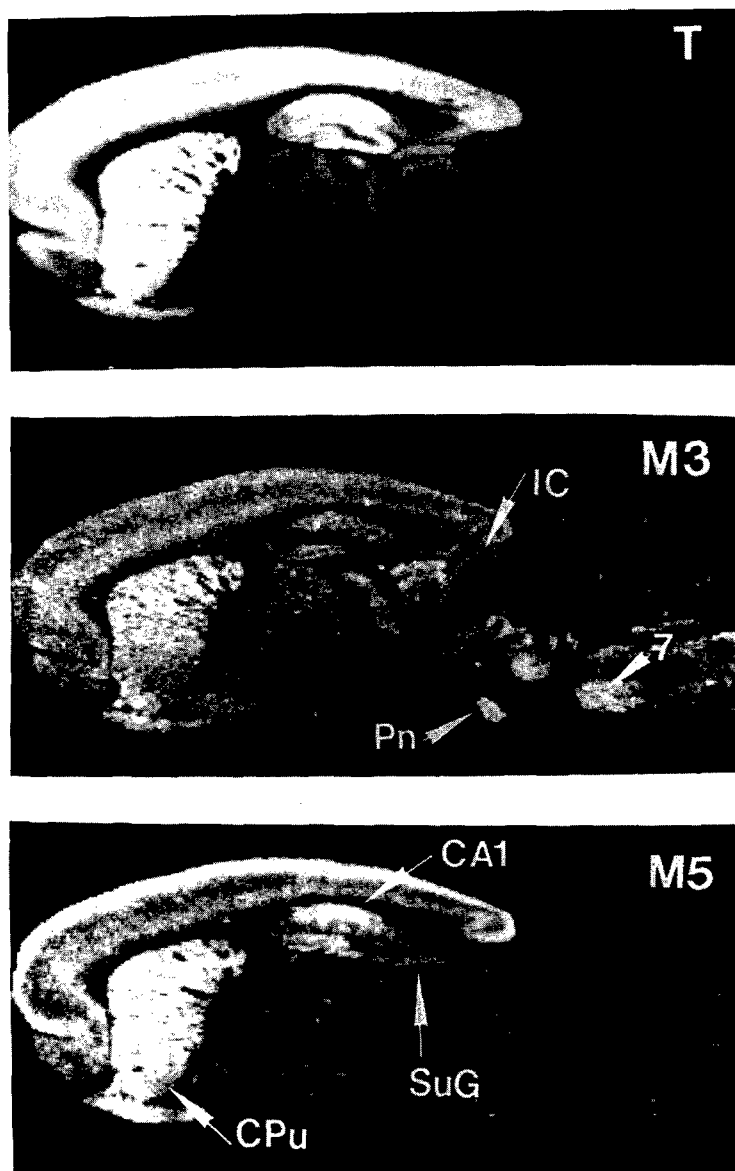


Figure 2. Grayscale images of autoradiograms of total (T), m3 and m5 muscarinic receptors in sagittal sections (2.4 mm lateral) of rat brain (20). Computer-generated grayscale coding was generated from scanned autoradiograms (*white*, highest densities; *gray*, intermediate densities; *black*, lowest densities). The range of optical densities was the same for the m3 and m5 autoradiograms (0-3.9) but expanded (0-20) to compare the distribution of the more abundant total muscarinic receptor population. *IC*, inferior colliculus; *Pn*, pontine nuclei; *7*, nucleus of the facial nerve; *CA1*, CA1 subfield of Ammon's horn; *SuG*, superficial gray layer of the superior colliculus; *Cpu*, caudate-putamen.

of both dopaminergic cell bodies in the SN/VTA and terminals in the striatum, respectively. While m5 labeling was lost in the SN/VTA region, there was little or no decrease in m5 labeling in the striatum. This result suggested that only a minor fraction of the m5 receptors in the striatum are on terminals of dopaminergic neurons originating in the SN/VTA, while the majority are on intrinsic or afferent striatal neurons. Due to the limits of resolution of light microscopic autoradiography, the identification of the specific population of neurons that express the m5 receptors in the striatum awaits the development of an m5-selective antibody.

Conclusions: What More Do We Know?

Selective labeling conditions for the M5 (m5) receptor subtype have been developed that have permitted for the first time autoradiographic localization studies of the m5 receptor in the brain. These m5-selective labeling conditions have assisted in studies of m5 receptor distribution in brain regions and peripheral tissues using *in vitro* test tube binding assays (19). The m5 receptor was enriched in the outer layers of the cortex, certain subfields of the hippocampus, caudate putamen, and olfactory tubercle. The availability of m5 receptor labeling conditions also has provided the first opportunity to directly compare the regional distribution patterns of all five muscarinic receptor subtypes. The distribution of the m5 receptor was distinct from the m3 and other muscarinic receptor subtypes. The relative levels of expression of the m5 receptor appeared to be higher and more widespread than previously anticipated from *in situ* hybridization studies. Results from lesioning experiments have provided evidence for the expression of the m5 receptor on both cell bodies and terminals of dopaminergic neurons in the SN/VTA, as well as on other intrinsic striatal neurons. While the studies described here represent a small step forward in our understanding of the M5 (m5) receptor subtype, it is anticipated that they will enhance interest and further study of this forgotten muscarinic receptor subtype. The development of m5-selective ligands and antibodies and identification of tissues and/or cell lines that express the native m5 receptor protein are necessary in order to determine the functions and physiological significance of the m5 muscarinic receptor subtype.

Acknowledgments

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MUSCARINIC REGULATION OF THE L-TYPE CALCIUM CURRENT IN ISOLATED CARDIAC MYOCYTES

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Summary

Muscarinic agonists regulate the L-type calcium current in isolated cardiac myocytes. The second messengers pathways involved in this regulation are discussed briefly, with particular emphasis on the involvement of cAMP and cGMP pathways.

Key Words: heart, cardiomyocyte, acetylcholine, isoprenaline, GTP-binding protein, adenylyl cyclase, cyclic AMP, cyclic GMP, nitric oxide, guanylyl cyclase, phosphodiesterase, calcium channel

Although intensively studied over the last decades, the cardiac effects of acetylcholine (ACh) are still not clearly described at the molecular level. In the whole heart, ACh exerts a direct negative chronotropic effect on the sino-atrial node, a negative dromotropic effect on atrio-ventricular conduction, a direct negative inotropic effect on the atria, and indirect negative inotropic and lusitropic effects which are observed both in atrial and ventricular tissues in the presence of a sympathetic tone (34,55,58). When used at concentrations $>10 \mu\text{M}$, muscarinic agonists exert an additional positive inotropic effect (34). In isolated cardiac myocytes, activation of the muscarinic receptors by ACh modifies the activity of second messenger pathways, ionic channels, contractile proteins and calcium homeostasis (34,55,58,91,92). These effects are attributed to the M2 receptors based on the use of selective ligands of the muscarinic receptors subtypes. However, functional M1 and M4 receptors are also expressed in cardiac myocytes of some animal species (23,91,101). The identity of the second messengers involved in the muscarinic regulation in cardiac myocytes is a field of controversy (34,47,58). To illustrate this purpose, we will briefly review the muscarinic regulation of the cardiac L-type calcium current.

The L type calcium current (I_{Ca}) is a major determinant of the plateau phase of the action potential and is the trigger of contraction in cardiac myocytes (see 34). Therefore, its regulation by sympathetic and parasympathetic systems and their respective neuromediators, noradrenaline and ACh, is most relevant for cardiac physiology. Since the muscarinic regulation of I_{Ca} consists essentially of an anti-adrenergic effect, it is necessary to first summarize the β -adrenergic regulation of I_{Ca} . Beta-adrenergic agonists, such as isoprenaline, produce a stimulation of I_{Ca} in all types of cardiac myocytes studied so far (34,38). Activation of β -adrenergic receptors stimulates the stimulatory GTP-binding protein (G_s protein), which in turn enhances cAMP production by adenylyl cyclase (18,20,34,38,58). The increase in cytoplasmic cAMP level activates the cAMP-dependent protein kinase (cA-PK) which is ultimately responsible for the increase in I_{Ca} (20,26,34,84). Similarly, adenylyl cyclase activation is involved in the stimulation of I_{Ca} by histamine (42,52), prostaglandin I_2 (2), parathyroid hormone (95,105,106), relaxin (30), serotonin (81), glucagon (66), VIP (100) and CGRP (74). Direct activation of adenylyl cyclase with forskolin

also leads to an increase in I_{Ca} , (3,19,35,37,75,82,83,109) which is not mediated by activation of a G protein (19,35,37,82, 83). Whether cAMP generation accounts for all the β -adrenergic stimulation of I_{Ca} has been a matter of debate (38,108). However, most reports have now demonstrated that cA-PK inhibitors fully antagonize the β -adrenergic stimulation of I_{Ca} , which confirms that cAMP is the only relevant second messenger involved in this regulation (38,82,83).

Muscarinic agonists reduce and/or eliminate the β -adrenergic stimulation of I_{Ca} (14,36, 41,43). They also antagonize the stimulatory effects of histamine (58), prostaglandin I_2 (2), serotonin (81), glucagon (18), CGRP (74) and forskolin (37,38,51,75,80). In marked contrast, the stimulation of I_{Ca} by intracellular perfusion with exogenous cAMP or non hydrolyzable analogs of cAMP is not reduced by muscarinic agonists (9,14,29,75,80,82,83,85). This suggests that the locus of action of ACh is prior to cAMP generation. Besides, in microperfusion experiments, ACh was found to inhibit the isoprenaline-stimulated I_{Ca} only in the part of the cell superfused by both muscarinic and β -adrenergic agonists (45). This suggests that the inhibitory effect of ACh on I_{Ca} does not require a diffusible second messenger. The inhibitory effect of ACh (or carbachol, CCh) on I_{Ca} is prevented by pertussis toxin (9,75,80). The cardiac substrates of the toxin are the G proteins, G_o and G_i (20,34,97). G_i has been shown to antagonize the activation of adenylyl cyclase by G_s . The α_i subunit of the G_i proteins has been demonstrated to be the relevant subunit involved in the inhibition the type V and VI adenylyl cyclase, the cardiac isoforms. However, the muscarinic inhibition of I_{Ca} can be altered either by the selective inhibition of α_i (51) or by internal dialysis with $\beta\gamma$ complexes (9). Thus, the relative role of α_i and $\beta\gamma$ subunits in the muscarinic regulation of I_{Ca} is not fully understood. Surprisingly, in frog atrial myocytes, pertussis toxin injection antagonizes the effect of nanomolar ACh concentrations on I_{Ca} , but it does not fully abolish the inhibition of I_{Ca} by micromolar concentrations of ACh (56). This is observed even when the activation by ACh of the muscarinic potassium current, $I_{\text{K,ACh}}$ is totally suppressed by pertussis toxin. However, the pertussis toxin-resistant effect of ACh is still mediated by G proteins, since it is abolished by intracellular GDP β S. Intracellular application of non hydrolyzable GTP analogs (GTP γ S and GppNHp) mimics, in an irreversible manner, the inhibitory effect of ACh on isoprenaline- or forskolin-stimulated I_{Ca} (19,75,80,82,83). Like ACh, these analogs were found to have no effect on the cAMP-stimulated I_{Ca} . Altogether, these data suggest that the muscarinic inhibition of I_{Ca} is due entirely to the inhibition of adenylyl cyclase.

In the absence of β -adrenergic stimulation, i.e. under « basal » conditions, ACh does not modulate I_{Ca} unless the adenylyl cyclase is under a tonic activation. This can be tested by investigating the effects of cA-PK inhibitors or phosphodiesterase (PDE) inhibitors on I_{Ca} . Indeed, if a basal activation of adenylyl cyclase is responsible for maintaining a baseline stimulation of I_{Ca} , then cA-PK inhibitors or PDE inhibitors will respectively inhibit or stimulate basal I_{Ca} . In the cells where such a behavior is found, ACh is also able to reduce the basal I_{Ca} (rabbit sino-atrial myocytes, 85), or to antagonize the stimulation of I_{Ca} by PDE inhibitors (ventricular myocytes, 54,72). This inhibitory effect of ACh is also pertussis toxin-sensitive (23,72,85) and, thus, likely results from a G_i -inhibition of basal adenylyl cyclase. What is the reason for the presence of a significant basal activation of adenylyl cyclase in some cells and not in others? Most likely the relative proportion of G_i vs. G_s , and/or of β -adrenergic vs. muscarinic receptors. Indeed, spontaneous activation of G_s and G_i by empty receptors has been unveiled in intact cardiac myocytes by the inverse agonistic effects of β -adrenergic and muscarinic receptors, respectively (44,63,64). Thus, β -adrenergic antagonists, such as atenolol and propranolol (70), and muscarinic antagonists, such as atropine and AF-DX 116 (33), were found, respectively, to inhibit and stimulate I_{Ca} in the absence of any receptor agonist. Moreover, the spontaneous activation of $I_{\text{K,ACh}}$ by GppNHp was significantly slowed down by muscarinic antagonists (33). Therefore, the basal effect of ACh on I_{Ca} , like that of PDE- or cA-PK-inhibitors, may depend on which of the inhibitory G_i - and stimulatory G_s -pathways dominate at rest.

The results summarized above are consistent with the hypothesis that ACh inhibits I_{Ca} via a reduction of cAMP production. This conclusion is supported by some biochemical data (24,34,50,57,58), but not all. Other studies showed that ACh produces inhibitory effects on contraction and I_{Ca} without any detectable changes in cAMP levels or cA-PK activity (7,25,61,76,77,87,111). In some of these studies, the effect of ACh was attributed to the stimulation of a phosphatase activity (1,25,40,57,76,77). Although this mechanism may participate in the overall effect of ACh, the lack of detectable changes in cAMP concentration at the cellular level does not necessarily exclude an inhibition of adenylyl cyclase as part of this effect (57). Indeed, cAMP changes may occur in discrete pools near the sarcolemmal membrane which would make them undetectable by classical means. Such local increases in cAMP, which lead to local elevations of I_{Ca} , have been shown to occur in isolated frog ventricular myocytes during the activation of β -adrenergic receptors (45,46). In these experiments, application of ACh to the part of the cell not exposed to the β -adrenergic agonist has no effect on I_{Ca} . Thus, activation of phosphatase activity can not alone provide a satisfactory mechanism for the muscarinic regulation of I_{Ca} .

In addition to the muscarinic inhibition of I_{Ca} , seen in the nanomolar to micromolar range of ACh concentration, higher concentrations of agonist produce a stimulation of I_{Ca} . (23,34). This stimulation occurs via a pertussis toxin insensitive pathway. Indeed, in guinea-pig ventricular myocytes, CCh enhances I_{Ca} after stimulation of the current by intracellular cAMP and this effect is increased after treatment of the myocytes with pertussis toxin (23). This stimulatory effect on I_{Ca} likely involves activation of M1 receptors, and may participate in the pirenzepine-sensitive increase in intracellular free calcium concentration and contraction induced by CCh (91, but see 92). The likely mechanism for this effect is activation of a pertussis toxin-insensitive G protein, leading to activation of phospholipase C, and activation of protein kinase C/IP₃ pathways.

More than twenty years ago, and in many studies since then, muscarinic agonists were shown to increase cGMP levels in the heart (21,34,58,59,61). The participation of this cyclic nucleotide in the effects of ACh on cardiac myocytes is still a matter of debate. One difficulty comes from the fact that a significant fraction of the cardiac cGMP production occurs in smooth muscle cells, where it participates in the relaxation of coronary vessels by ACh (22, 102). Cyclic GMP levels were also found to be raised by ACh or CCh in cell suspensions enriched with cardiomyocytes, suggesting that cGMP could play a role in the muscarinic regulation of cellular functions in these cells (11,47,49,59,61). A soluble guanylyl cyclase activating factor was found to be secreted by cardiac myocytes in the presence of muscarinic agonists (4). This factor could be nitric oxide (NO) since its production is abolished by treating the cells with L-arginine analogs, which are NO-synthase (NOS) inhibitors, and is restored by adding an excess of L-arginine, the natural NOS substrate (4). Furthermore, cardiac myocytes express the constitutive endothelial NOS (eNOS or NOS3), and can express the inducible NOS (iNOS or NOS2) under certain circumstances (4,5,13,47,89,90). However, in one study, the elevation of cGMP levels induced by ACh was also found to be insensitive to L-arginine analogs suggesting that factors other than NO may link ACh to cGMP production (94).

Since I_{Ca} is strongly regulated by the cGMP/NO pathway, it is tempting to speculate that this pathway participates in the muscarinic regulation of I_{Ca} . Intracellular application of cGMP or activation of soluble guanylyl cyclase activity by NO-donors produce stimulatory and/or inhibitory effects on I_{Ca} depending on the concentrations used and/or on the animal species (reviewed in 59). Stimulation of I_{Ca} may result from an increase in cAMP concentration consecutive to the inhibition by cGMP of PDE3, a cGMP-inhibited PDE (15,16,17,48,78,79, 95,103). Inhibition of I_{Ca} may involve either a reduction of cAMP level due to the activation by cGMP of PDE2, a cGMP-stimulated PDE (17,36,59,68,69,78,93,95), or the activation of the cGMP-dependent protein kinase (cG-PK) (27,53,59,79,96,98,99,103,104,107 but see 28). Modification of functional amino

acid residues may lead to additional effects on I_{Ca} with some NO-donors used at high concentrations ($>100 \mu\text{M}$) (8). Like ACh, the regulation of I_{Ca} by cGMP takes place only in the presence of an elevated intracellular cAMP concentration. However, unlike ACh, cGMP and NO-donors regulate I_{Ca} even when the adenylyl cyclase activity is bypassed by intracellular application of cAMP (15,17,36,53,59,68,69,79). The simplest explanation of this result is that ACh acts upstream and the cGMP/NO pathway downstream from cAMP generation. Thus, this pathway, if turned on by ACh, may represent a secondary mechanism of regulation that could play a modulatory role in the overall effect of ACh on I_{Ca} .

Surprisingly, in several recent studies performed in rabbit SA node (31,32) and AV node cells (29) and in rat ventricular myocytes (5), L-arginine analogs were found to fully antagonize the muscarinic inhibition of I_{Ca} , suggesting an obligatory role for NO in this regulation. In some of these studies, L-arginine could restore the effect of muscarinic agonists on I_{Ca} after application of the analogs (29,31,32). In cat atrial myocytes, application of a NOS inhibitor was shown to remove the rebound stimulation of I_{Ca} that follows ACh washout (107). Furthermore, methylene blue and LY 83583, two putative guanylyl cyclase inhibitors (10,73, 88), were shown to reduce the inhibition of I_{Ca} by ACh or CCh (5,29,31,32,54,72). While these studies would support the hypothesis that the activation of the NO/cGMP pathway takes a major part in the inhibitory effect of muscarinic agonists on cardiac I_{Ca} (reviewed in 47), several other studies do not. For instance, in frog ventricular myocytes, the inhibition of I_{Ca} by ACh is totally insensitive to L-arginine analogs in spite of the fact that cGMP and NO-donors produce clear effects on I_{Ca} (36,67-69). In guinea-pig ventricular myocytes, the muscarinic inhibition of the cAMP-stimulated chloride current is also totally resistant to L-arginine analogs (110). Furthermore, the results of the experiments with methylene blue should be interpreted with caution since this compound inhibits the muscarinic activation of $I_{\text{K,ACh}}$ which is clearly not mediated by the NO/cGMP pathway (32,43). A recent study of ours demonstrates that methylene blue acts as a muscarinic receptor antagonist (43). Moreover, LY 83583 and methylene blue are superoxide anion generators and, as such, can modify the redox equilibrium of the cells (6,60,62, see also 10,12,65,71). For instance, LY 83583 potentiates the stimulatory effect of isoprenaline on I_{Ca} , even in the absence of guanylyl cyclase activity (43, see also 110). Thus, at this stage, the issue of the participation of the NO/cGMP pathway in the regulation of I_{Ca} by ACh remains unsettled.

In summary, while inhibition of adenylyl cyclase was considered for many years as the only mechanism responsible for the muscarinic inhibition of cardiac I_{Ca} , several other mechanisms have come out recently, most particularly activation of phosphatase and activation of NO/cGMP pathway. However, the participation of these additional mechanisms varies greatly (from all to none) in different published studies. These discrepancies need to be clarified, and most particularly the issue of possible animal species and/or cardiac tissue differences. Other questions that will need to be examined include: i) the characterization of the mechanisms involved in the coupling of muscarinic M2 receptors to eNOS (pertussis toxin sensitive G protein? Ca_i^2 ?), ii) the potential role of NO in the muscarinic inhibition of adenylyl cyclase, iii) the respective roles of each mechanism (adenylyl cyclase, phosphatase, NOS) in the overall effect of ACh, and iv) the targets of NO and/or cGMP leading to ACh regulation of I_{Ca} .

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ACTIVATION OF M₂ MUSCARINIC RECEPTORS IN GUINEA-PIG ILEUM OPENS CATIONIC CHANNELS MODULATED BY M₃ MUSCARINIC RECEPTORS

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Summary

In longitudinal muscle of guinea-pig ileum, activation of muscarinic receptors causes contraction antagonised by M₃ receptor subtype antagonists despite a preponderance of M₂ receptor subtype binding sites. Experiments on single smooth muscle cells under voltage-clamp described here show that the cationic current evoked by carbachol which normally causes depolarization of the muscle is inhibited competitively by M₂ antagonists with affinities typical of antagonism at a M₂ receptor. However, M₃ antagonists strongly reduced the maximum cationic current which could be evoked by carbachol in a non-competitive manner with affinities typical for an action at M₃ receptors. Thus cation channels are gated by M₂ receptor activation but strongly modulated by activation of M₃ receptors.

Key Words: M₂ receptor, M₃ receptor, cationic current, guinea pig ileum, smooth muscle

Activation of the muscarinic receptors of longitudinal smooth muscle of guinea-pig small intestine produces an increased frequency of action potential discharge and depolarization which results in contraction. The contractions evoked are generally regarded as mediated via the M₃ subtype of muscarinic receptor (1) although the muscle has a preponderance of M₂ subtype muscarinic binding sites (2) which appear to be non-functional. The explanation of this enigma is at present unclear. It is generally believed that M₁ and M₃ subtypes link preferentially G_q/G₁₁ G-protein and phospholipase C, inositol trisphosphate formation and calcium store release while M₂ and M₄ receptors link preferentially to G_i/G_o Pertussis toxin-sensitive G-protein and inhibition of adenylate cyclase (3). The effects of activating M₂ receptors in this muscle can be demonstrated under special condition for example where adenyl cyclase activity has been stimulated with forskolin or by β -adrenoceptor activation (4). In other experiments the M₃ receptor was inactivated with 4-DAMP mustard (4-diphenylacetoxy-N-(2-chloroethyl) piperidine) while M₂ receptors were largely protected with a suitable concentration of the M₂ antagonist methoctramine. Following this both histamine (which contracts) and isoprenaline (which inhibits this contraction) were applied. Under these conditions M₂-mediated contractions to oxotremorine M were observed (5).

The depolarization produced by acetylcholine or carbachol (6;7) is caused by the opening of channels which admit sodium and allow potassium to escape from the cell (8;9). Calcium under normal conditions probably does not pass through these channels in significant amounts (*cf* 9 with 10). With high concentrations of carbachol (50-300 μ M) producing a presumed high fractional receptor occupancy, the current-voltage relationship of the evoked cationic current is linear from 0 mV to -50 mV or more negative (8;11). At more negative potentials the cationic current does not increase linearly with driving force so that the current-voltage relationship is U-shaped (inward

current plotted downwards). The potential at which deviation from linearity occurs is more negative the higher the concentration of carbachol and moves progressively less negative as desensitisation occurs (11;12). Modulation of I_{cat} occurs with variations in internal free ionised calcium, $[Ca^{2+}]_i$ (13;14). This seems to occur because the cationic channels opened by muscarinic receptor activation are sensitive to $[Ca^{2+}]_i$ (e.g. 15).

Methods

Single smooth muscle cells were obtained by digestion of small fragments of longitudinal muscle pulled gently off the small intestine of the guinea pig immediately after euthanasia (11). Using patch pipettes made of borosilicate glass (resistance 1-4 M Ω) gigaseals were formed onto the membrane of single cells and whole-cell recording mode was obtained by rupturing the underlying on-cell membrane patch with pulses of suction.

Cells were voltage clamped and the current required is displayed in the records. Cells were held at -40 mV and after stepping to 0 mV a negative-going ramp at $33\frac{1}{3}$ mV/sec was applied to -40 mV. This ramp was applied when current was steady before and during the application of carbachol at various concentrations to the cell to activate muscarinic receptors. After digitalisation current before carbachol was subtracted from that in the presence of carbachol. The resulting current-voltage relationship, (or its linear portion if at negative potential deviations from linearity occurred) was fitted with a linear regression; the slope of this was the activated conductance carrying the cationic current, I_{cat} . In the figures this conductance is plotted against carbachol concentration on a logarithmic scale.

It was necessary to remove several other obfuscating currents and complicating mechanisms. Inside the cell and in the bathing solution there was sodium- and potassium-free 124 mM cesium chloride; the Cs^+ ion does not readily pass through potassium channels but moves freely through cationic channels. In fact using this symmetrical cesium-based solution results in a larger than normal cationic current which improves the signal to noise ratio of the recording. Calcium and magnesium ions were not added to the bathing solution as their presence reduces I_{cat} (16). Also, as I_{cat} is very sensitive to increases in the free calcium concentration on the inner side of the membrane (13;14), $[Ca^{2+}]_i$ was "clamped" at 10^{-7} M by use of a 10 mM BAPTA/4.6mM $CaCl_2$ combination. The use of these conditions produces a large cationic current carried exclusively by cesium and not subject to modulation by changes in $[Ca^{2+}]_i$ in the bulk cytoplasm; it is however still feasible that local microdomains exist inside the cell where $[Ca^{2+}]$ can change in response to muscarinic receptor activation.

In some experiments sodium replaced cesium in the internal and external solutions and 1.2 mM $MgCl_2$ and 2.5 mM $CaCl_2$ were added to the external solution. Sodium is also quite impermeant through potassium channels but I_{cat} under these conditions is much smaller and the signal-to-noise ratio is less good (Zholos & Bolton, 1996b).

Results

Carbachol was applied by changing the solution bathing the cell to one containing it; ascending concentrations were applied which evoked inward current (Fig. 1A). When this was steady, conductance was estimated from a negative going ramp from 0 mV to -40 mV after leakage subtraction. It should be noted that this method avoids any effects of the shift of the activation curve with changes in fractional receptor occupancy or desensitisation (16). The conductance

TABLE 1
Muscarinic Antagonists pA₂ Values

Functional studies

	Atropine	Methoctramine	Tripitramine	Himbacine	4-DAMP	HHSD	Zamifenacin
1	9.28	6.77			9.04	7.28	
2	8.27	5.36			8.64	7.84	
3	8.72			7.47	9.41	6.94	
4	8.60			7.54	9.09	7.44	
5							9.31
6	9.1	5.3		7.0	9.4	6.7	7.6
7	9.4	5.5				7.2	8.2
8	9.0	7.1	cAMP	Inhibition	(M ₂)	6.5	
9		6.35	6.81				
10		6.30	6.34				
11	9.0						
12	8.15					7.92	
13	10.29	6.10			8.29	7.31	
14		6.17				7.84	

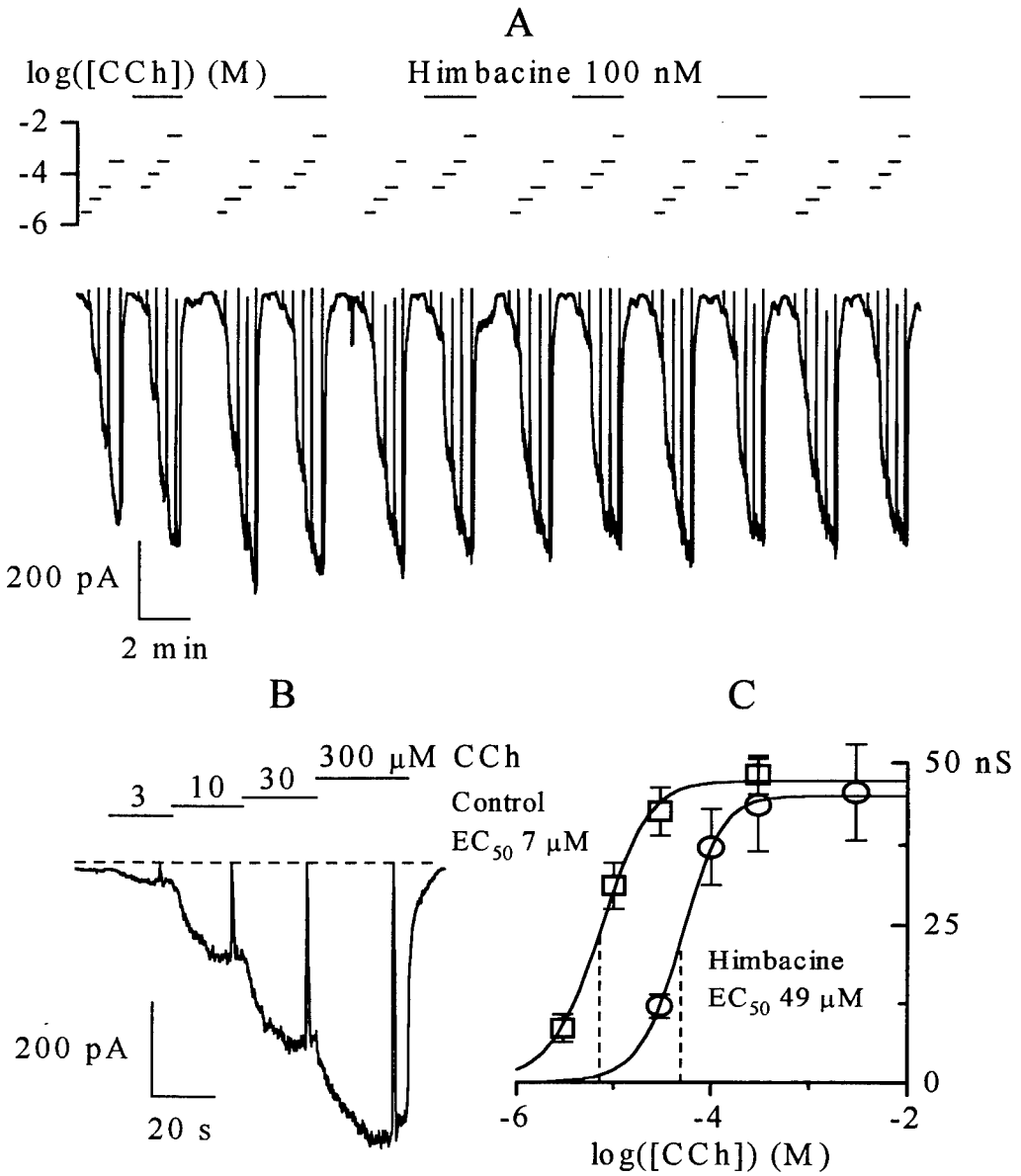
Binding studies

	Atropine	Methoctramine	Tripitramine	Himbacine	4-DAMP	HHSD	Zamifenacin
1		7.84 (m2) 6.56 (m3)	9.57 (m2) 7.42 (m3)				
2	8.82					6.04	
3		6.68			7.42		
4		6.15		8.17	7.71		
5		8.89				7.17	
6	8.69	7.56			8.29		
7		7.88 (m2) 6.67 (m3)		8.0 (m2) 7.03 (m3)	8.42 (m2) 9.28 (m3)	6.88 (m2) 7.81 (m3)	

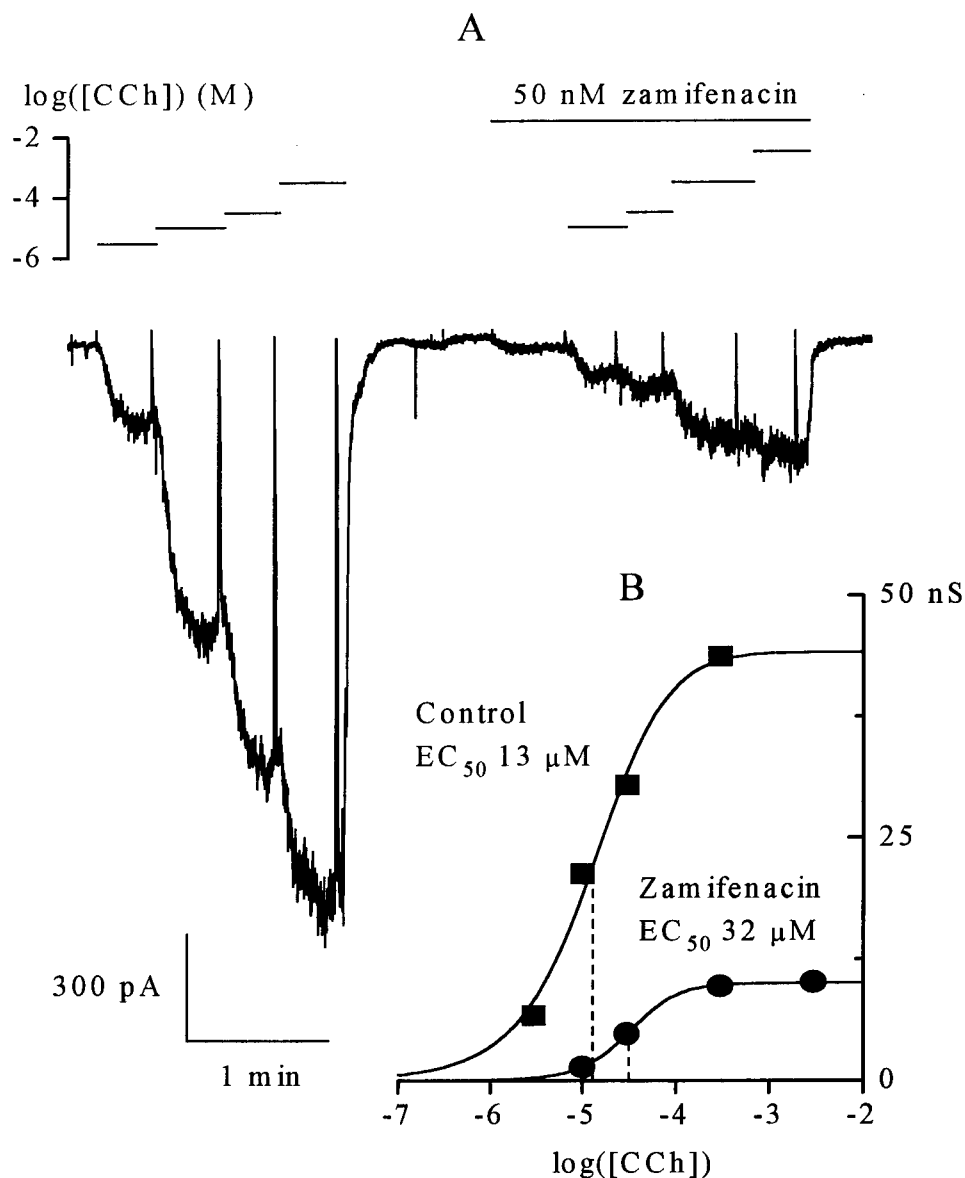
Our study

	9.00	8.11	9.10	7.97	7.36	7.16	7.94
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In functional studies the pA₂ was obtained from the inhibition of contractile responses of the tissue; in binding studies subpopulations of binding sites were distinguished as indicated. **References for functional studies** 1. Rat urinary bladder (23); 2. Rabbit urinary bladder (24); 3. Circular muscle human colon (25); 4. Longitudinal muscle human colon (25); 5. Guinea-pig ileum (26); 6. Human bronchial smooth muscle (27); 7. Guinea-pig trachea (28); 8. Guinea-pig ileum (5); 9. Guinea-pig ileum (29); 10. Guinea-pig trachea (29); 11. Guinea-pig ileum (30); 12. Rabbit cecum (31); 13. Single ileal smooth muscle cells (32); 14. Guinea-pig ileum (1); **References for binding studies** 1. Cloned human m2 receptor (33); 2. Rabbit cecum (31); 3. Canine colonic circular muscle (34); 4. Rat duodenum (35); 5. Guinea-pig ileum (36); 6. Longitudinal muscle guinea-pig ileum (36); 7. Cloned human receptors (37).

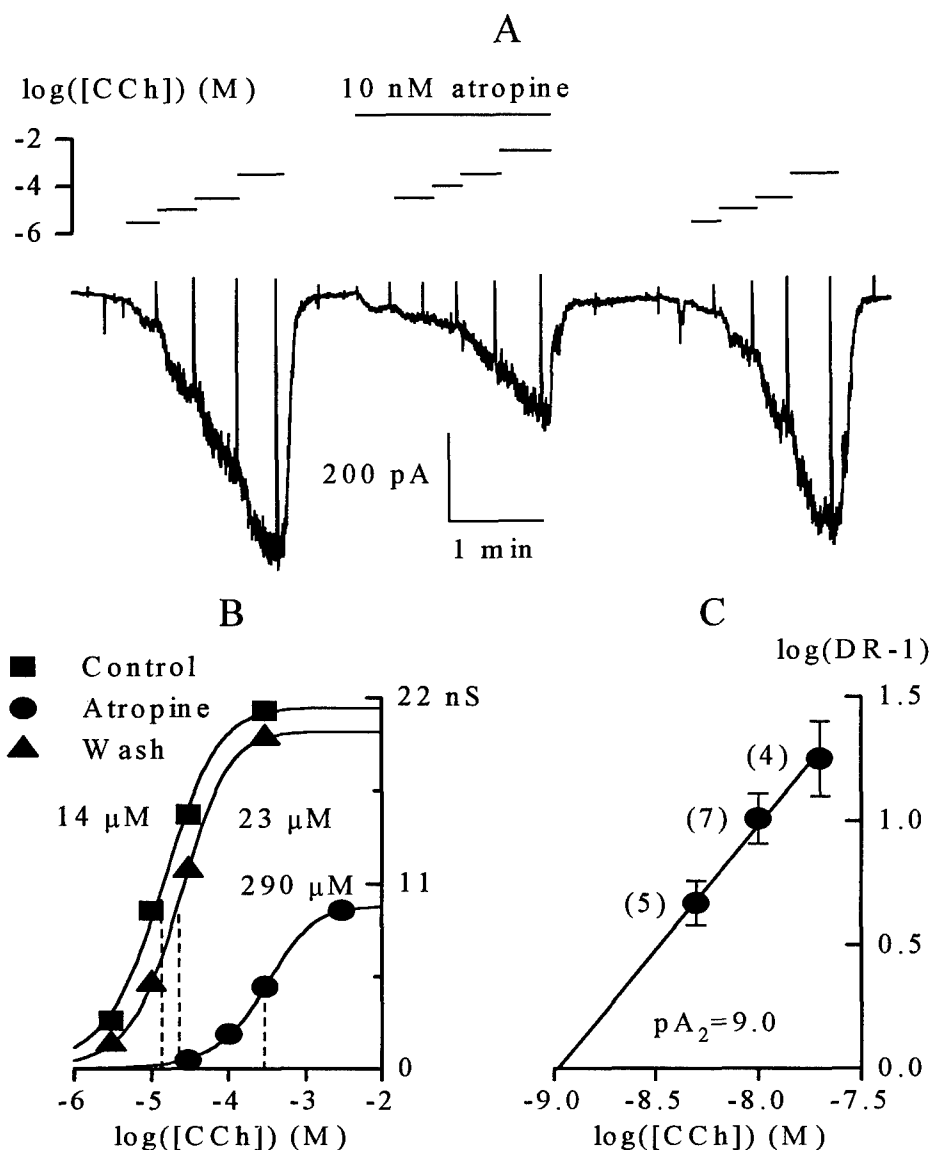
**Fig. 1**

Himbicine antagonism of carbachol (CCh) inward current in single voltage-clamped smooth muscle cell held at -40 mV. **A.** Twelve series of ascending CCh concentrations (bars; refer to left-hand log scale) were applied; note increase in concentration in himbicine (100 nM). **B.** Inward current in response to ascending series of CCh concentrations on expanded time base; vertical deflections represent ramp applied to determine current-voltage relationship. **C.** Himbicine (100 nM) effect on CCh concentration-conductance relationship; points are means of 6 determinations \pm s.e.m. Dotted lines mark EC_{50} s; ordinate is conductance in nanosiemens.

**Fig. 2**

Zamifenacin antagonism of CCh inward current. A. Series of ascending concentrations of CCh applied to single cell held at -40mV to evoke inward current. B. Zamifenacin (50nM) severely reduces maximum response and shifts CCh concentration-conductance curve slightly to right. See figure 1 and methods for further details.

opened by muscarinic receptor activation was plotted against carbachol concentration; by choosing four suitable concentrations the position of the concentration-effect curve could be accurately obtained (Fig. 1B). Repeated application of the ascending series of carbachol concentrations produced some increase in the EC₅₀ in a series of control cells and a small reduction in the maximal effect of carbachol but neither was sufficient to interfere with measurements of the effects of antagonists.

**Fig. 3**

Atropine antagonism of inward current response in single voltage-clamped cell held at -40mV. **A.** Atropine (10nM) reduces maximum inward current to CCh. **B.** Atropine (10nM) reduces maximum response and shifts CCh concentration-conductance curve to right; EC₅₀ concentrations are shown. **C.** Schild plot using EC₅₀ values obtained in 4-7 cells.

M₂ antagonists. Three selective M₂ antagonists were investigated and results can be illustrated by the action of himbacine (Fig. 1). At 100 nM himbacine a rightward parallel shift of the concentration-effect curve was obtained (Fig. 1C); only one concentration of himbacine was applied to each cell. The effects of himbacine were rapidly reversible upon washing and could be repeated several times in the same cell (Fig. 1A). No decline in the maximal response was

observed compared to control experiments. Three concentrations of himbacine were used (5×10^{-8} M, 10^{-7} M and 2×10^{-7} M) on 3-9 cells and a Schild plot constructed. The slope was not significantly different from unity. The pA_2 value was 7.97. Experiments where sodium replaced cesium in pipette and bathing solution, and calcium and magnesium were added to the bathing solution, gave similar values. Tripitramine and methoctramine gave pA_2 values of 9.10 and 8.11 respectively (Table 1).

M₃ antagonists The actions of these can be illustrated by the effects of zamifenacin. Zamifenacin (5×10^{-8} M) severely depressed the maximum response (Fig. 2A, about 75%) without much effect on the EC_{50} of the concentration-effect curve to carbachol (Fig. 2B). Higher concentrations produced even more depression of the maximum, and shifted the concentration effect curve to the right with increase in the EC_{50} . Two other M₃ blockers 4-DAMP (4-diphenylacetoxy-N-methylpiperazine methiodide) and HHSD (p-fluoro-hexahydro-siladiphenidol) behaved similarly.

Atropine which acts at both M₂ and M₃ receptors both depressed the maximum response (M₃ effect) and shifted the concentration-effect curve to the right (M₂ effect) (Fig. 3A,B,C). Analysis of such a situation is difficult; if dose-ratios based on EC_{50} s were obtained by scaling the curves depressed in the presence of antagonist, then Schild analysis gave pA_2 values of 7.36 (4-DAMP) 7.17 (HHSD) and 7.94 (Zamifenacin). These were close to those expected for binding of these antagonists to M₂ rather than M₃ receptors.

Discussion

The parallel shift of the concentration effect curve for M₂ antagonists himbacine, methoctramine and tripitramine implied that the cation current is gated by activation of a M₂ receptor. It is known that the cationic current response is Pertussis toxin sensitive indicating a G_o/G_i link to channel opening (17,18). However, M₃ blockers all reduced severely the maximum response of which cells were capable. This result implied that M₃ receptor activation exerts a potent modulatory effect on cationic channel opening in an entirely unexpected way.

It is difficult to be certain about the mechanism of this effect. It could represent an interaction at the level of G-protein between M₃ and M₂ subtypes. One possibility is that M₃ activation is necessary for some process such as phosphorylation of the M₂ receptor or cationic channel and if this is reduced by blocking the M₃ receptors then cation channel opening is severely impaired; this could be interpreted as a "permissive" effect of M₃ activation on M₂ gating of cationic channel. Such phosphorylations as have been described so far seem likely to suppress rather than enhance receptor function (e.g. 19;20). A further possibility is that the calcium buffer (BAPTA-CaCl₂) does not "clamp" [Ca²⁺]_i in a microdomain between subplasmalemmal sarcoplasmic reticulum calcium stores and plasma membrane. This small crevice, much smaller than 0.1μ (21;22) may be inaccessible to BAPTA and [Ca²⁺]_i may rise normally when M₃ receptors are activated due to PLC activation, inositol trisphosphate formation and Ca-store release into this crevice. Since a rise in [Ca²⁺]_i strongly potentiates the cationic current, M₃ receptors may by this mechanism effectively control the size of the cationic current gated by M₂ receptor activation. Blocking M₃ receptors may seriously attenuate the M₂-cationic channel link.

Acknowledgement

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TOLTERODINE - A NEW BLADDER SELECTIVE MUSCARINIC RECEPTOR ANTAGONIST: PRECLINICAL PHARMACOLOGICAL AND CLINICAL DATA

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Summary

Tolterodine is a new, potent and competitive muscarinic receptor antagonist in clinical development for the treatment of urge incontinence and other symptoms of unstable bladder. Tolterodine has a high affinity and specificity for muscarinic receptors *in vitro* and it exhibits a selectivity for the urinary bladder over salivary glands *in vivo*. A major active metabolite, (PNU-200577) the 5-hydroxymethyl derivative of tolterodine, has a similar pharmacological profile. Based on pharmacological and pharmacokinetic data, it has been concluded that this metabolite contributes significantly to the therapeutic effect of tolterodine. The bladder selectivity demonstrated by tolterodine and PNU-200577 *in vivo* cannot be attributed to selectivity for a single muscarinic receptor subtype. Moreover, this favourable tissue-selectivity seems to occur also in humans. Tolterodine is well tolerated and it exerts a marked effect on bladder function in healthy volunteers. Phase II data indicate that tolterodine is an efficacious and safe treatment for patients with idiopathic detrusor instability or detrusor hyperreflexia. An optimal efficacy/side-effect profile is obtained with tolterodine, at a dosage of 1 or 2 mg twice daily, which seems to have less propensity to cause dry mouth than the currently available antimuscarinic drugs.

Key Words: urinary bladder, incontinence, dry mouth, *in vivo*, human

Urinary incontinence is reported by 5-10% of the adult population and the prevalence, particularly of urge incontinence, increases with age (1). The symptoms of an unstable bladder comprise urge incontinence, urgency and frequency. Unstable bladder is presumably caused by uncontrolled detrusor contractions during the filling phase. It is generally agreed that contractions of the human bladder are mediated mainly by cholinergic muscarinic receptors (2). The bladders of various species, including humans, contain a mixed population of muscarinic M_2/m_2 and M_3/m_3 receptors (3). The M_2/m_2 receptors predominate, but it is generally believed that the contractile response is mediated only by the M_3/m_3 receptors (3). The pharmacological treatment of unstable bladder has for many years been based on muscarinic receptor antagonists (2) and oxybutynin is currently the drug of choice. The effectiveness of oxybutynin has been documented in several controlled clinical studies, but its usefulness is limited by classical antimuscarinic side-effects, which often leads to discontinuation of treatment (4). Dry mouth, for example, is experienced by at least 50% of patients treated with oxybutynin (4).

Tolterodine (PNU-200583, (R)-N,N-diisopropyl-3-(2-hydroxy-5-methylphenyl)-3-phenylpropan-amine) is a new muscarinic receptor antagonist under clinical development for the treatment of unstable bladder. In this paper, the antimuscarinic *in vitro* and *in vivo* profiles of tolterodine and a major active metabolite (the 5-hydroxymethyl derivative, PNU-200577, Labcode DD 01) are reviewed in comparison to those of oxybutynin and some other selective muscarinic receptor antagonists. Clinical data on tolterodine from phase I studies in healthy volunteers and from phase II studies in patients with detrusor hyperreflexia and idiopathic detrusor instability are also presented.

Preclinical pharmacology

Tolterodine is a potent and competitive inhibitor of carbachol-induced contractions of isolated urinary bladder preparations from guinea pig (KB 3.0 nM) (5) and humans (KB 4.0 nM) (6) and it binds with high affinity to muscarinic receptors in the bladder (Table I) and other tissues (5). Tolterodine does not exhibit any selectivity with respect to the human muscarinic receptors, expressed in Chinese hamster ovary (CHO) cells. The K_i values determined at m1-m5 receptors are (nM): 3.0, 3.8, 3.4, 5.0 and 3.4, respectively (7) (c.f. Table I). The *in vitro* profile of the 5-hydroxymethyl metabolite of tolterodine (PNU-200577), is similar to that of tolterodine (8).

Although M_2/m_2 receptors predominate in the urinary bladder of various species, bladder contraction is considered to be mediated by the minor population of M_3/m_3 receptors (3). This hypothesis is supported by functional *in vitro* data determined in the guinea pig urinary bladder (KB, Table I) (7) for the selective reference compounds darifenacin (M_3 selective) (9), UH-AH 37 (low affinity for M_2/m_2) (10) and AQ-RA 741 (M_2/m_2 selective) (11). On the other hand, the data on oxybutynin do not fit into this scheme. Oxybutynin shows a distinct selectivity profile with respect to the human m1-m5 receptors expressed in CHO-cells, with the highest affinity at m3 receptors (K_i 0.67 nM) > m4, m1 (K_i 2.0 and 2.4 nM) \geq m2 (K_i 6.7 nM) \geq m5 (K_i 11 nM) (7), but the KB-value derived from studies on bladder contraction (4.4 nM) obviously correlates with binding data at m2, rather than at m3 receptors (Table I).

Table I

Functional (KB) and Binding (K_i) Data in Guinea Pig Tissues and CHO-Cells

Drug	KB (nM)		Ki (nM)		
	Urinary Bladder	Urinary Bladder	Parotid Gland	m2	m3
Tolterodine	3.0	2.7	4.8	3.8	3.4
PNU-200577	0.84	2.9	5.2	2.0	2.5
Oxybutynin	4.4	4.0	0.62	6.7	0.67
Atropine	0.70	1.6	0.85	2.9	1.0
Darifenacin	0.87	78	1.7	56	1.2
UH-AH 37	5.2	82	26	49	7.2
AQ-RA 741	140	12	170	4.4	55

Data from refs. 5, 7- 8.

Tolterodine and PNU-200577 have about 8 times lower affinity than oxybutynin in the parotid gland (M_3) and 4-5 times lower affinity at m_3 receptors in CHO-cells (Table I) but they are not less potent than oxybutynin in inhibiting carbachol-induced contractions of the guinea pig bladder (5) (Table I). PNU-200577 is in fact 5 times more potent than oxybutynin in this respect. This cannot be explained by additional action(s) at other cellular targets, since both tolterodine and PNU-200577 show a very high degree of specificity for muscarinic receptors (5, 8 and Nilvebrant, unpublished data). Moreover, both tolterodine and oxybutynin effectively inhibit electrically induced contractions of human detrusor strips, with IC_{50} values (tolterodine: 2.5 nM, oxybutynin: 3.2 nM) that are similar to the K_B - and K_i - values determined in guinea pig bladder (12). The lack of correlation between functional data on bladder contraction and binding affinity at M_3/m_3 receptors is not unique for oxybutynin. A similar profile has been found for dicyclomine: the K_B value determined for dicyclomine in guinea pig bladder is 24 nM, (Nilvebrant, unpublished data), while the K_i -values determined in heart (M_2) and parotid gland (M_3) are 24 nM and 2.8 nM, respectively (13). Whether these data indicate that not only M_3/m_3 receptors are involved in bladder contraction (14, 15) or that M_3 receptors may be heterogeneous (16), remains to be clarified.

Table II
Selectivity Profiles *In Vivo* and in *In Vitro* Binding Studies

Drug	Anaesthetised cat ID 50 nmol/kg		Affinity ratios <i>in vitro</i>		
	Bladder Contraction	Salivation	Selectivity	Guinea pig Tissues Bladder/Parotid gland	CHOcells m_2/m_3
Tolterodine	101	257	Bladder	0.6	1.1
PNU-200577	15	40	Bladder	0.6	0.8
Oxybutynin	200	104	Salivary gland	6.5	10
Atropine	18	21	Non-selective	1.9	2.9
Darifenacin	119	99	Salivary gland	46	47
UH-AH 37	311	120	Salivary gland	3.2	6.8
AQ-RA 741	1060	1536	Bladder	0.07	0.08

Data from refs. (5, 7, 8, 17). Bladder contraction *in vivo* was induced by acetylcholine (i.a.) and salivation was induced by electrical stimulation of the chorda-lingual nerve. Antagonists were administered by intravenous infusion. Affinity ratios were calculated from the K_i -values displayed in Table I.

The most interesting feature of tolterodine and PNU-200577 is that they are significantly more potent in inhibiting urinary bladder contractions than salivation in the anaesthetised cat (17, 8) (Table II). This favourable selectivity profile can obviously not be attributed to selectivity for a single muscarinic receptor subtype. However, the reference compounds which in binding studies show a selectivity for parotid gland over bladder and for m_3 over m_2 receptors in CHO-cells, oxybutynin, darifenacin and UH-AH 37, are more effective in inhibiting salivation than bladder contraction *in vivo* (Table II). Thus, a selectivity for M_3/m_3 over M_2/m_2 receptors is not necessary for an effective inhibition of bladder contraction *in vivo*, but may result in a more pronounced effect on salivation (Fig. 1).

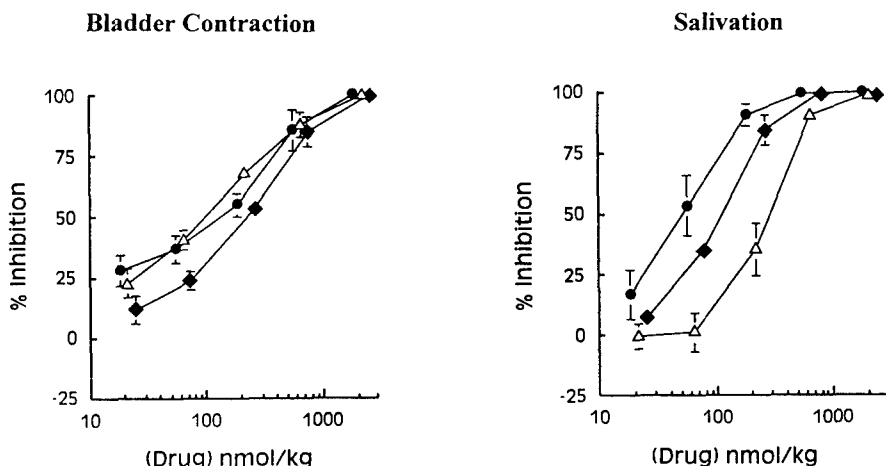


Fig. 1

Effects on urinary bladder contraction and salivation in the anaesthetised cat. The rank order of potency for tolterodine (Δ), oxybutynin (\blacklozenge) and darifenacin (\bullet) is different with respect to these two responses. Plots constructed from data in (7).

The mechanism behind the bladder selectivity demonstrated for tolterodine and PNU-200577 *in vivo* is not known. Based on the combined *in vitro* and *in vivo* data, it may be speculated either that the M_3/m_3 receptors in glands are more sensitive than those in the bladder, or that the M_3 receptors are heterogeneous (15). On the other hand, the M_2/m_2 selective antagonist AQ-RA 741 exhibits a bladder selectivity which, although less pronounced, is similar to that demonstrated for tolterodine and PNU-200577 (7). This may suggest that M_2/m_2 receptors are involved in bladder contraction (c.f. 14-16) and that blockade of M_2/m_2 receptors in the bladder contributes to the favourable selectivity profiles of tolterodine and PNU-200577. However, muscarinic receptors involved in transmitter release are present on both adrenergic and cholinergic nerves in the detrusor of different species (2). A mixed prejunctional population of inhibitory M_2 and facilitating M_1 receptors has been demonstrated on postganglionic cholinergic nerves in the detrusor (18). The regulation of bladder function is complex and the relative functional importance of the different muscarinic receptors *in vivo* remains to be clarified. Nevertheless, the bladder selectivity demonstrated for tolterodine and PNU-200577 in the preclinical pharmacological *in vivo* studies seems to occur also in humans, as indicated by the data from the clinical phase I and II studies presented below.

Clinical pharmacokinetics/pharmacology

Tolterodine is rapidly absorbed and an approximate dose-proportional increase in peak serum levels is observed after about 1 h (19). Basic pharmacokinetic parameters are: half-life 2-3 h, systemic clearance about 30 l/h and volume of distribution about 110 l. The major metabolic pathway of tolterodine involves hydroxylation of the 5-methyl group, mediated by cytochrome P450 2D6, resulting in the 5-hydroxymethyl derivative PNU-200577 (19-21), which is

pharmacologically active (8). Similar mean peak serum concentrations (C_{max}) of tolterodine and PNU-200577 are found in most human subjects (20). The 5-hydroxymethyl metabolite is further metabolised to the corresponding carboxylic acid. The other primary metabolic pathway, which is of minor quantitative importance, involves N-dealkylation of tolterodine (21). Excretion of the 5-carboxylic acid and the N-dealkylated 5-carboxylic acid metabolites accounts for about 80% of the dose excreted in urine. The excretion of intact tolterodine is low (<1%) (19).

In the first clinical phase I study, tolterodine was given as single oral doses (0.2–12.8 mg) to healthy volunteers (19). After 12.8 mg, heart rate was increased by 35% after 1 h and salivary secretion (induced by chewing of paraffin wax) was decreased. Long-lasting (until the next day) and marked effects on bladder function, such as micturition difficulties, were reported by the healthy volunteers (22). Effects on the bladder were noticed also when dry mouth was no longer reported. This was the first indication that the selectivity for bladder over salivary glands is present also in humans. In a later study (23), in which effects on bladder function were measured by cystometry, it was confirmed that tolterodine exerts a significant inhibitory effect on bladder function after a single oral dose of 6.4 mg. Stimulated salivary secretion was also inhibited, but only around C_{max} , while the effects on the bladder were more persistent. Thus, significant effects on cystometric variables were registered 5 h after dose and one subject experienced micturition difficulties up to 10 h after dose. No significant effects on blood pressure or heart rate were found. However, a considerable volume of residual urine was observed (23).

One subject was found to have significantly higher serum levels of tolterodine than the other volunteers, without showing divergent urodynamic effects. It was speculated that this subject was a poor metaboliser of tolterodine (23). Later, in the clinical phase II studies, the majority of patients in the four dose groups (0.5, 1, 2 and 4 mg) were found to have median peak serum concentrations of tolterodine of 0.4, 0.7, 1.5 and 3.8 $\mu\text{g/l}$, respectively, and concentrations of the 5-hydroxymethyl metabolite PNU-200577 in the same range (20). However, a small proportion of the patients showed a pharmacokinetic profile in accordance with poor metabolisers, having about ten times higher tolterodine concentrations but no measurable concentrations of the 5-hydroxymethyl metabolite. The unbound fraction of the metabolite in human serum is, however, 10-fold higher than for tolterodine (3.7%) (Påhlman and Gozzi, manuscript in preparation) and, together with the data on the antimuscarinic potency *in vitro* (8), this indicates that the 5-hydroxymethyl metabolite (PNU-200577) accounts for the major part of the pharmacological effect of tolterodine in extensive metabolisers. In Caucasians, it can be expected that about 7% of the population are poor metabolisers.

The conclusion from the early clinical phase I studies was that tolterodine shows a good tolerability over a wide dose-range. There was, however, an increase in urinary residual volumes after a dose of 6.4 mg and, therefore, 4 mg was selected as the highest dose for the clinical phase II studies. Despite the relatively short half-lives of tolterodine and PNU-200577, a twice daily dosage regimen was chosen because of the long-lasting effects on bladder function.

Clinical phase II studies

The safety, tolerability and efficacy of tolterodine have been evaluated in four randomised, double-blind, placebo controlled, parallel group, dose-ranging, multi-centre phase II studies. Patients with idiopathic detrusor instability (24) were recruited to two of these studies, while the

other two studies comprised patients with detrusor hyperreflexia (25-26). Data from the entire phase II programme, encompassing 319 patients (203 women and 116 men, mean age 51 years, range: 17-76), have been pooled for analysis of safety and efficacy (20). A frequency of at least 8 micturitions per 24 h, or at least one incontinence episode per 24 h, or both, was required for inclusion. A two-week wash-out period, during which any antimuscarinic treatment was prohibited, preceded randomisation. The treatment period was 14 days. Patients were randomised to one of four dosages of tolterodine tartrate: 0.5 mg bd ($n=70$), 1 mg bd ($n=62$), 2 mg bd ($n=65$), 4 mg bd ($n=58$), or placebo ($n=64$). The criteria for a per protocol evaluation of efficacy was fulfilled by 262 of the 319 randomised patients.

Effects on the bladder were objectively measured by cystometry. Significant dose-effect relationships were found for the volume at first contraction, the maximum cystometric capacity and residual volume (Fig 2) (20). The volumes at first sensation, normal desire to void, and strong desire to void also increased significantly in a dose-dependent manner.

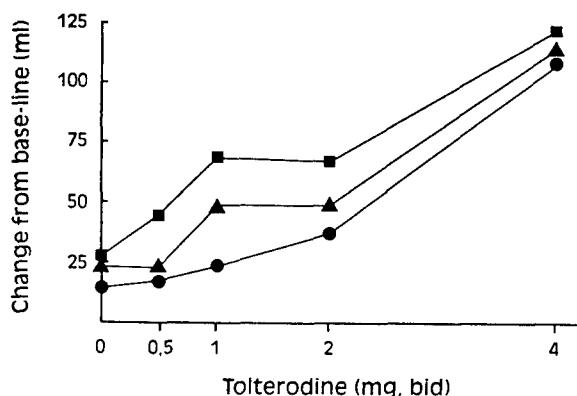


Fig. 2

Effect of tolterodine on cystometric variables measured in clinical phase II studies in patients with idiopathic detrusor instability or detrusor hyperreflexia: volume at first contraction (■), maximum cystometric capacity (▲) and residual volume (●), after two weeks of treatment.

Urinary symptoms, recorded in micturition charts, showed a dose-dependent improvement in frequency of micturition, average volume voided and number of incontinence episodes (20, 24-26). In the groups treated with tolterodine 2 or 4 mg bd, the frequency of micturition decreased by about 15% and the average volume voided increased by 24% (20). The number of incontinence episodes decreased by 38% (2 mg bd) and 46% (4 mg bd). Many of the patients with detrusor hyperreflexia were on clean intermittent self-catheterisation (25-26) and their habits cannot be expected to change during the rather short treatment period of two weeks. The effects on micturition chart variables may therefore be underestimated in the pooled material. Thus, in one of the studies on patients with detrusor instability (24), incontinence episodes decreased by 60-65% and the frequency of micturition by 21-27%.

There were no safety concerns with respect to blood pressure, clinical chemistry or haematology variables (20, 24-26). A minor, dose-dependent, increase in heart rate was noted (average 6 beats/

min at tolterodine 4 mg bd) but there were no clinically significant changes in QT interval, QTc or other ECG variables (20). Dose-reduction was allowed during the first week of treatment, but this possibility was only used by nine patients. Five of these were in the 4 mg bd group (Table III). The number of withdrawals was lower for tolterodine than for placebo. Eighteen of the patients treated with tolterodine ($n = 255$) were tentatively classified as poor metabolisers. The tolerability and efficacy profile in these patients did not differ from that in the other patients.

Table III

Withdrawals and Dose-reductions

Treatment	Dose (mg bd)	Patients (n)	Dose-reductions (n)	Withdrawals (n)
Placebo	0	64	0	5
Tolterodine	0.5	70	1	2
Tolterodine	1	62	3	2
Tolterodine	2	65	0	2
Tolterodine	4	58	5	3
<hr/>				
Total		319	9	14

Tolterodine was well tolerated and the adverse events reported were mainly of an antimuscarinic nature. Dry mouth was reported by 13% (8/64) of the patients in the placebo group. In the tolterodine groups, dry mouth was reported by 7% (5/70) of the patients on 0.5 mg, 13% (8/62) on 1 mg, 26% (17/65) on 2 mg and by 36% (21/58) of the patients on 4 mg (20). The dry mouth was considered mild to moderate and the incidence seems to compare favourably with reported data on oxybutynin (4). However, five cases of urinary retention occurred, four in the 4 mg bd group and one in the 2 mg bd group. Together with the marked increase in residual volumes observed in the 4 mg bd group, this indicates that a dosage of 4 mg bd is too high and, thus, that the optimal efficacy/side-effect ratio is achieved with tolterodine in dosages of 1 or 2 mg bd (20, 24-26). These doses were therefore selected for the phase III clinical programme.

Conclusions

Tolterodine is a potent muscarinic receptor antagonist which exhibits a favourable selectivity for the urinary bladder over salivary glands *in vivo*, in the anaesthetised cat. A major, active metabolite, the 5-hydroxymethyl derivative of tolterodine (PNU-200577), has a similar profile. The bladder selectivity of tolterodine and PNU-200577 *in vivo* cannot be attributed to muscarinic receptor subtype selectivity. However, data from clinical phase I and II studies indicate that a favourable selectivity profile is also obtained in humans. Tolterodine is well tolerated and it exerts marked inhibitory effects on bladder function while the propensity to cause dry mouth seems to be less pronounced. Phase II data on tolterodine in patients with idiopathic detrusor instability or detrusor hyperreflexia indicate that an optimal efficacy/side-effect ratio is achieved with tolterodine in dosages of 1 or 2 mg bd.

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MUSCARINIC MECHANISMS IN NERVE CELLS

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Summary

The receptor subtype and transduction mechanisms involved in the regulation of various neuronal ionic currents are reviewed, with some recent observations on sympathetic neurons, hippocampal cell membranes and basal forebrain cells.

Key Words: potassium channels, calcium channels, G proteins, messengers, muscarinic subtypes

Muscarinic receptor (mAChR) activation provokes a variety of effects on different nerve cells. These may be characterized loosely as 'excitatory' or 'inhibitory' (though with caveats, see below). 'Excitatory' effects result principally from closure of one or more of a number of different K^+ channels [see (1) for a recent review], though instances of cation channel opening have also been described [e.g. (2,3,4)]. Effects which may be classed as 'inhibitory' include opening of K^+ channels and closure of voltage-gated Ca^{2+} channels. Assuming that there are some general rules that govern the way in which each receptor subtype can potentially affect each type of ion channel, this variability might depend on the relative abundance of the different mAChR subtypes, the nature of the ion channels, and the presence of the appropriate components of the receptor-channel transduction machinery in each cell. Various aspects of this general question have been reviewed in recent years (5-12). The ensuing account summarizes some of the more salient points arising from recent work.

A. Potassium channels closed by mAChRs

1. K_M channels ('M-channels').

Inhibition of these channels 'de-represses' neurons, to increase excitability and facilitate repetitive spike discharges (e.g., 13,14). This is the principal cause of mAChR-induced excitation of some sympathetic neurons (the 'slow epsp'); and, along with inhibition of 'SK' K_{Ca} channels (see below), contributes to cholinergic excitation of cortical and hippocampal neurons. [It should be noted that, where mAChR-sensitive K_M and K_{Ca} channels are co-expressed, inhibition of one will enhance the repressive influence of the other, so full de-repression requires inhibition of both currents (13,15)].

Receptor subtypes. M-channels are preferentially inhibited by m1(M1) or m3(M3) receptors. This has been most clearly shown using NG108-15 cells transformed to express different recombinant mAChR subtypes at 'physiological' levels (0.2-0.8 pmoles/mg.protein) (16,17). [m5-

receptors have not been tested.] Receptors mediating $I_{K(M)}$ inhibition in rat superior cervical sympathetic ganglia have been identified pharmacologically as M1 [pirenzepine pK_B 7.5 (18) and 8.0 (19); AF-DX 116 pK_B 6.0 (18); himbacine pK_B 6.9 (19)]. These neurons also express mRNA for m3 receptors (1) but receptor protein levels may be low (20). Receptors responsible for $I_{K(M)}$ inhibition in central neurons have not been fully characterized but appear to be less sensitive to pirenzepine (21,22); while originally taken to suggest the involvement of M2 receptors, this would also be compatible with M3 receptors.

G-proteins. mAChR-induced $I_{K(M)}$ inhibition is resistant to *Pertussis* toxin (PTx). Experiments using antibodies directed against α -subunit C-terminal sequences suggest that $I_{K(M)}$ inhibition in rat sympathetic neurons is mediated (at least in substantial part) by G_q and/or G_{11} (23). Subsequent studies using expression plasmids to drive production of antisense RNA complementary to the 3'-untranslated regions specific to the different α -subunits (24) has characterized G_q as the responsible member of this pair (25). However, the same approach has also revealed a contribution by another G-protein, G_{12} , to $I_{K(M)}$ -inhibition (26,27) - interesting because, unlike G_q , G_{12} is not known to couple to PLC. It is not yet known whether the α subunit or its attached $\beta\gamma$ subunits act as the transducer.

Second messenger(s)? Although the kinetics of M-current inhibition may be compatible with a rather 'direct' coupling between the active G-protein subunit and the channel (28) [but see also (29)], there is fairly compelling evidence from on-cell patch-clamp recording to suggest a more 'remote' signalling mechanism, involving a diffusible (?intracellular) messenger (30-32). However, the identity of this messenger (or messengers) is still uncertain. Clearly, the identification of one of the activated G-proteins as G_q points in the direction of phospholipase C (PLC) products; this is reinforced by the fact that both of the effective mAChR subtypes [and other receptors which 'couple' to M-channels, such as tachykinin and bradykinin receptors (13,33)] strongly activate PLC.

One candidate pathway for PLC-mediated transduction is diacylglycerol(DAG)-mediated protein kinase C (PKC) activation. Although $I_{K(M)}$ is frequently inhibitable by PKC-activators, mAChR-induced inhibition has usually proved resistant to PKC antagonists (34,35) [but see (36,37)]. Nevertheless, two recent developments have led us to investigate this possibility a little further. First, the analogous 'remote' inhibition of inward rectifier K^+ channels in forebrain neurons by the PLC-activator, substance P may be mediated by PKC (38). Second, we have identified a prominent Ca^{2+} /PKC-activated Cl^- current in rat sympathetic neurons which can clearly be induced or potentiated by stimulating M1-mAChRs (39). Using this response as a 'control', we find that procedures which prevented the induction of the Cl^- current by mAChR agonists (such as prior treatment with 100 μM calphostin-C or 1 μM bisindolylmaleimide) did not affect mAChR-induced M-current inhibition (40). Hence, we agree that PKC is unlikely to mediate $I_{K(M)}$ -inhibition by muscarinic agonists [see also (41)], though this does not rule out a more direct action of diacylglycerols (42).

The obvious alternative (or conjunctive) pathway is through the formation of inositol triphosphate and subsequent elevation of intracellular $[Ca^{2+}]$. It is clear from recent experiments on excised membrane patches that M-channels can be readily inhibited by rises in intracellular $[Ca^{2+}]$ - either directly [in rat ganglion cell membranes (43)] or through activation of the Ca^{2+} /calmodulin-dependent phosphatase calcineurin [in frog ganglion cell membranes (44); the two are not necessarily in conflict]. However, the extent to which such effects may be responsible

for mAChR-induced M-channel closure is still uncertain [see (43-45) for recent assessments of the relevant experimental data].

2. 'SK' (K_{Ca}) channels.

These are the 'small' [low-conductance: ~ 2 pS in asymmetric $[K^+]$ (46)] Ca^{2+} -activated K^+ channels (K_{Ca} channels) which are widely distributed in peripheral and central neurons and which generate long post-spike after-hyperpolarizations ('AHPs' - hence sometimes dubbed 'AHP channels'). Inhibition of these channels, with consequent 'de-repression' of spike discharges, contributes a major component to the excitatory action of synaptically-released acetylcholine in the brain, particularly in the hippocampus where it has been most extensively studied [see (47)]. In enteric neurons, the mAChR responsible for this inhibition is blocked by pirenzepine, with a K_B of ~ 10 nM (48), so is probably M1. This may also be true for the hippocampal receptor (22), though no K_B values are available. A complication for receptor sub-typing is that the AHP-current can also be reduced if the priming Ca^{2+} current which initiates SK channel opening is reduced. The mAChR which does this is likely to be m2 or m4 (see below). This effect is not responsible for AHP-inhibition in the hippocampus, since the priming Ca^{2+} transient is unchanged (49), but is probably responsible for mAChR-induced AHP-inhibition in sympathetic ganglia (1). In keeping with this, ganglionic SK channels are preferentially activated by Ca^{2+} entry through the M4-sensitive N-type Ca^{2+} channels (50). The subsequent pathway to SK $_{Ca}$ inhibition following mAChR activation is not at all clear but is likely to be rather indirect, since synaptic inhibition of I_{AHP} has a minimum delay of >400 ms (51). Although the channels can be closed by activating PKC, this does not seem to be responsible for the effect of mAChRs since concentrations of PKC inhibitors which prevent the effects of PKC activators (or of cAMP, which also closes the channels) do not affect mAChR-induced inhibition (52). It has been suggested that it might result from activation of CaM kinase II (i.e., downstream to the IP_3 -mediated release of Ca^{2+}) since inhibition was prevented by injecting a peptide inhibitor protein (53) but the experiments are difficult to interpret because the peptide inhibitor itself appeared to reduce the AHP. An alternative possibility might be Ca^{2+} -dependent activation of a tyrosine kinase, since tyrosine kinase inhibitors block the comparable inhibition of the AHP-current produced by stimulating 'metabotropic' glutamate receptors (54). Two members of this family of K_{Ca} channels have recently been cloned (55): both contain a number of phosphorylation sites, so multiple regulatory mechanisms may exist.

3. 'Inward rectifier' potassium channels (K_{IR}).

mAChRs inhibit an inwardly-rectifying component of resting K^+ current in (e.g.) nucleus accumbens neurons (56) and in rat sympathetic neurons (57), probably via M1 receptors [pirenzepine K_B 11 nM in n. accumbens (56); pK_B for pirenzepine and himbacine 8.1 and 6.9 respectively in ganglia (57)]. The mechanism is unknown but the comparable effect of substance P in basal forebrain neurons appears to be mediated by a PKC-dependent mechanism (38). Recombinant m1 mAChRs also inhibit the cloned inward rectifier channel IRK1 (Kir2.1) expressed in mammalian tsA cells by a PKC-dependent mechanism (58): this may be relevant to the above effects because Kir2.1 and other members of this family are widely expressed in the brain (59) and in sympathetic ganglia (57).

4. 'Delayed rectifier' current.

A delayed rectifier current in ventromedial hypothalamic neurons is partially-inhibited by carbachol (60). The receptor subtype has not been clearly defined but inhibition was selectively reduced by injecting an antisense nucleotide to $G_{\alpha 11}$; unlike $I_{K(M)}$ (see above), antisense to $G_{\alpha q}$ was ineffective.

5. Fast-gating M-like currents.

Two muscarine-sensitive sustained voltage-sensitive K^+ currents which show some resemblance to M-currents but which activate and deactivate much more rapidly have recently been described in granule cells of the Islands of Calleja (61,62) and of the cerebellum (63). The mAChR responsible for inhibition of the latter current (dubbed $I_{K(SO)}$) is probably m3 and may involve Ca^{2+} since inhibition is dependent on the presence of extracellular Ca^{2+} (63).

6. 'Leak' currents.

Inhibitory effects of mAChR stimulation on resting 'leak' K^+ currents have been described in a number of cells, including hippocampal pyramidal cells, possibly through M1 (22) or M3 (64) receptors. We have recently detected Ba^{2+} -inhibitable resting K^+ channels of 12.2 pS slope conductance in asymmetric $[K^+]$ in excised patches from hippocampal cell membranes which may form a component of this 'leak' current (65). These were inhibited by internal Ca^{2+} with an IC_{50} of 118 nM. In on-cell patches, channel activity inhibited by applying muscarine to the extra-patch membrane and was enhanced in a Ca^{2+} -free external solution: this suggests a possible common transduction mechanism for both 'leak' and M-channel closure [cf. (30,43)].

7. Some cloned channels.

Several cloned channels known to be present in the mammalian nervous system can be closed by m1-mAChRs when expressed in appropriate cells. While the extent to which they participate in the *in situ* effects of mAChRs is not always clear, studies on such systems can provide valuable mechanistic insights. One example (the Kir2.1 inward rectifier channel) has been referred to above. Two other channels may be particularly pertinent to proposed mechanisms of inhibition of M, SK and SO channels - Kv1.2 of the *Shaker* family, and *reag*, a mammalian homologue of the *Drosophila ether-a-go-go* channel.

Kv1.2. This channel subunit protein is widely (though heterogeneously) distributed in rat brain (66) and is also present in NG108-15 cells (67,68). When expressed as a homo-oligomer it forms a dendrotoxin-sensitive channel which is inhibited by m1-mAChR activation (in oocytes) and bradykinin (in NG108-15 cells), and also by increasing intracellular $[Ca^{2+}]$ or stimulating PKC (68-71). This appears to result from a Ca^{2+} (or PKC)-induced phosphorylation of the tyrosine kinase PYK2 and subsequent tyrosine phosphorylation of the Kv1.2 protein, and is prevented by the tyrosine kinase inhibitor, genistein (68,72), though additional direct effects of internal Ca^{2+} might contribute (71). This differs from M-channel inhibition in being much slower in onset and offset; also, M-current inhibition appears insensitive to genistein (35; C.E. Stansfeld, unpublished observations). However, activation of tyrosine kinases may contribute to mAChR-induced suppression of other K^+ -currents in central neurons.

Reag. This mammalian homologue of *eag* is also present in the brain (73). When expressed as a homo-multimer in mammalian HEK 293 cells, it generates K^+ channels of comparable conductance and pharmacology to M-channels and comparably-slow, low-threshold macroscopic currents, though with more complex kinetics (74). *Reag* channels are rapidly and reversibly inhibited by stimulating mAChRs: this is probably mediated by a rise in intracellular $[Ca^{2+}]$ since (a) inhibition closely parallels the associated rise and fall of intracellular $[Ca^{2+}]$, and (b) channels can also be blocked by ionomycin or (in isolated inside-out patches) by direct internal application of Ca^{2+} with an IC_{50} of ~67 nM (74).

B. Potassium channels opened by mAChRs.

1. Inward rectifier (Kir) channels.

Channels homologous to the cardiac G-protein-activated inward rectifier channels are expressed in the brain (59,75), probably formed from co-association of GIRK1 (Kir3.1) with Kir3.2 (76) or Kir3.4 (77). Their activation is probably responsible for that form of mAChR-induced increase in K^+ conductance mediated by M2 mAChRs [e.g. (78-81)] or by m4 receptors (82). By analogy with the cardiac channels (59), this is presumably mediated by a direct effect of $\beta\gamma$ subunits freed from activated PTx-sensitive α -subunits (76) though neither this, nor the identity of the specific α -subunit interacting with the mAChR, has yet been established for native neurons.

2. K_{Ca} channels.

In transformed NG108-15 neuroblastoma/glioma hybrid cells or fibroblasts, activation of recombinant m1 or m3 mAChRs generates a large K^+ current carried by K_{Ca} channels opened by IP_3 -released Ca^{2+} ions (16,83). Similar effects occur in transformed CHO cells following activation of m1, m3 or m5 receptors (84). m2 and m4 receptors are ineffective in these cells, but can induce a somewhat comparable response in NL308 neuroblastoma-fibroblast hybrids, albeit at much higher agonist concentrations and through a different (PTx-sensitive) G-protein (85). Virtually-identical responses (possibly mediated by M1 receptors) occur in hippocampal neurons following rapid application of acetylcholine provided measures are taken to prevent 'washout' of the components of the intracellular transduction machinery (86) so may contribute to the inhibitory actions of synaptically-released acetylcholine: this needs further evaluation.

C. Calcium current inhibition.

The neuronal (N-type) Ca^{2+} current of transformed NG108-15 cells is preferentially inhibited (via a PTx-sensitive G-protein) by activating recombinant m2 and m4 mAChRs (87). In rat sympathetic neurons, similar mAChR-induced PTx-sensitive inhibition of $I_{Ca(N)}$ is mediated by M4 receptors (19). In rat basal forebrain neurones a comparable effect on N, P, and Q/R (?) currents is mediated by m2/M2 receptors (88) and in rat striatal neurones by m2 and/or m4 receptors (89). These effects [and others in hippocampal cells (90) and sensory neurons (91)] are 'membrane-delimited' [cf. (12)]. By analogy with the comparable effects of noradrenaline, they might well be due to activation of G_{α_o} (23,92) and consequent liberation and channel modulation by associated $\beta\gamma$ subunits (93, 94). An additional 'remote' inhibition of both N-type and L-type Ca^{2+} channels has been detected following M1-receptor stimulation in sympathetic neurons (95,96). This is resistant to PTx and may be mediated by the same (unknown) messenger system as that which inhibits $I_{K(M)}$ (12). A similar PTx-resistant 'parallel-path' inhibition of the L-type current, possibly mediated by M1 receptors, has been reported in striatal neurons (97).

All of the above concerns observations on somatic high-threshold Ca^{2+} currents. Apart from the consequent inhibition of K_{Ca} currents (referred to earlier), their functional significance *vis-a-vis* somatic electrical activity is not entirely clear. However, since comparable channels are present on nerve terminals (98,99) [though with some variations: see (100)], a more interesting possibility is that inhibition of these Ca^{2+} currents might be responsible for the well-known auto-inhibitory effect of acetylcholine on its own release - for example, in the cerebral cortex. To explore this, we have recently assessed the effect of mAChR-stimulation on the action potential-evoked release of acetylcholine from individual release sites along the processes of dissociated cholinergic basal forebrain neurons in tissue culture, using a nicotinic channel 'sniffer-patch' to

obtain high spatial and temporal resolution (101). Muscarine (10 μ M) inhibited release by $\sim 70\%$. The sensitivity of the release process accorded with the sensitivity of the somatic Ca^{2+} current (IC_{50} 1.7 μ M) and appeared to be mediated by the same (M2) receptor as that responsible for I_{Ca} inhibition [cf. (88)] - according with hypothesis that presynaptic auto-inhibition in these cholinergic afferents results from inhibition of the terminal Ca^{2+} current. An alternative possibility - that it results from activation of a K^{+} current and terminal hyperpolarization [e.g. (102)] - is less likely because the cells we used showed no somatic response of this type. However, we cannot rule out additional intracellular effects 'downstream' to Ca^{2+} entry reported for some other presynaptic inhibitory transmitters (102-104).

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BEHAVIOURAL EVALUATION OF CHOLINERGIC DRUGS

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Summary

Alzheimer's disease involves progressive degeneration of the cortex and the limbic system. Loss of afferent forebrain neurochemical modulatory systems is also seen, most significantly of the basal forebrain cholinergic system. Drug discovery programmes have pursued enhancement of forebrain muscarinic function as a therapeutic target. The most promising muscarinic agonists described achieve functional selectivity as agonists as the M₁/M₃ receptors in the CNS and M₂ antagonists. These compounds have fewer cardiac and other cholinergic side effects. In rodent and monkey models of reference and working memory, these compounds reverse the cognitive impairment induced with plopalamine. Acetylcholinesterase inhibitors are even more efficacious in these models.

Key Words: muscarinic receptor, agonists, memory, cholinergic drugs

Cholinergic neurones are widely distributed in the brain and spinal cord (1). Acetylcholine (ACh) released at CNS synapses interacts with nicotinic receptors and with at least five classes of muscarinic receptors which have been cloned and characterised pharmacologically.

The distribution of the cholinergic projection pathways and of nicotinic and muscarinic receptors in the brain provides some insight to the behavioural pharmacologist seeking evidence of the functional significance of acetylcholine release in the brain. Cholinergic mechanisms are known to be important in the corpus striatum, where a balance between dopamine and ACh ensures normal motor output. Dysfunction of this balance occurs in Parkinson's disease and can be treated with anticholinergic drugs. Pain perception and temperature control also involve central muscarinic receptors. Muscarinic agonists raise pain thresholds and lower core temperature. These *in vivo* pharmacological responses can be measured usefully as an index of CNS penetration, potency and duration of action of muscarinic drugs during preclinical studies.

Since muscarinic agonists and antagonists have a range of CNS effects these may compromise behavioural efficiency. Side effects including salivation, gastrointestinal symptoms and cardiovascular effects are recognized to be of major significance in the efforts to develop muscarinic drugs for the treatment of Alzheimer's disease (AD).

The cholinergic system and cognition

The focus of this paper are studies of the role of ACh in forebrain systems involved in learning and memory which have acquired particular significance in relation to the understanding of AD and its treatment (2). AD is characterised by progressive degeneration of the neocortex and limbic system. The neuropathology is associated with the neuronal deposition of insoluble β -amyloid protein plaques and the formation of intra neuronal neurofibrillary tangles consequent upon abnormal processing of neuronal tau protein. In addition, monoamine containing neurones of the isodendritic core (3) show progressive degeneration; the retrograde loss of cholinergic neurones from the basal forebrain is the most common and the most severe neurochemical consequence of the disease. The cholinergic neurone clusters of the basal forebrain innervate the hippocampus and areas of association cortex involved in higher processes such as long term memory, working memory and attention. The severe impairment of memory seen in the early stages of AD are consistent with dysfunction of these brain systems. Following these discoveries, a great deal of animal and clinical research was undertaken to investigate the "cholinergic hypothesis" of AD; specifically that the cognitive impairments seen in AD are a consequence of forebrain cholinergic loss. The further implication of this hypothesis is that cholinergic replacement therapy, by analogy with the use of L-dopa in Parkinson's disease, may offer treatment for AD.

There is now a substantial body of experimental work in animals and man testing the concept that cholinergic mechanisms are essential for learning and memory. Drugs, such as scopolamine, which attenuates CNS cholinergic functions through non-selective blockade of muscarinic receptors or lesions of cholinergic neurones of the basal forebrain impair performance on a variety of learning and memory tasks. In a frequently cited human study, Drachman (4) demonstrated that in healthy young students, intra-muscular scopolamine impaired long term memory while leaving immediate memory intact. This deficit was reversed by physostigmine, a short-acting acetylcholinesterase inhibitor, which pharmacologically would be expected to enhance synaptic cholinergic function. A substantial literature now exists in humans and animals replicating the finding that cholinergic agonists can reverse the effects of scopolamine on long term memory tasks and that cholinergic lesions impair performance on such tasks. Under conditions of cholinergic deficiency previous experience no longer modifies future behaviour and a failure of memory storage is assumed. However, memory tasks can be failed just as readily if the relevant information is not attended to, or perceived, or if the response mechanism is deficient. Only recently, in an analysis of excitotoxin lesions of the cholinergic basal forebrain has it been appreciated that ACh is involved in various aspects of information processing (5). The earlier assumption that cholinergic manipulations influence only the storage of new information is incorrect. Efforts are being made to define the contribution of the various sectors of the forebrain cholinergic system to the different aspects of information processing. It is perfectly reasonable to use the existing drug and lesion models for evaluating cognitive enhancers, but caution must be exercised in assuming that storage of information rather than some other psychological process is being manipulated.

The goal is to develop in animals and humans a range of behavioural tasks which exploit the different components of information acquisition and storage likely to be compromised in AD.

It is also essential to include, as controls, measures of other aspects of behaviour not thought to involve cholinergic systems. These tasks might be totally unaffected by cholinergic manipulations or serve to evaluate non-specific adverse effects of cholinergic drugs on behaviour.

Animal models of the cognitive decline seen in AD

Cognition involves attention, encoding, memory, planning and thought. There is evidence that all of these processes are affected during the course of AD. However, most attention has focused on the striking impairment of long term memory seen early in the disease. The ability to hold information beyond the brief span of immediate memory is generally called long term memory but cognitive psychologists now recognise several distinct forms of this memory.

In working memory, an internal representation of information must be held for a short time and is task or trial specific. It is the information required from moment to moment to complete tasks satisfactorily. Reference memories are usually of longer duration and may be retrievable given an appropriate cue. For example, where personal items are stored in the house or what happened earlier in the day. Both forms of memory are impaired in AD and are believed to involve frontal and temporal lobe memory systems. Animal models attempt to measure the same memory processes but one must be cautious because, clearly, there are fundamental differences between animal and human cognition (6). Language, planning and thought are impossible to study in animals. However, a number of animal models of memory with face validity to the human equivalent have been useful for evaluating the cognitive enhancing effects of muscarinic agonists in rats and monkeys.

We have used passive avoidance and conditioned suppression of drinking as a measure of reference memory and delayed matching to position to assess drug effects on working memory.

1. Passive avoidance (PA) in rodents

PA continues to be a useful and widely used early screen. Typically it involves one-trial learning. The mouse or rat is placed in the brightly lit section of a two chamber box. The rodent has a strong natural tendency to pass into the darkened "safe" part of the box. Entry into the dark results in a mild electric shock to the feet. This event can be retained over extended periods of time the strength of the memory is measured by the reluctance (increased latency) of the animal to re-enter the dark box after a delay.

2. Conditioned suppression of drinking

Rats are trained over two days to drink a sweetened solution. On the 3rd day in the box, fluid is not available and the rat hears a series of tones and receives simultaneously a small electric shock to the feet. Under these conditions fear is classically conditioned to the tone. On day 4 drinking is re-established and on day 5, the latency to drink in the presence of the tone is measured. Normal rats are reluctant to initiate drinking but if treated with scopolamine on day 3, rats drink with a short latency on day 5. It is assumed that the ability to remember the conditioning experience on day 3 was impaired by the cholinergic antagonist.

3. Delayed matching or non-matching to spatial position

This task is tested in an operant chamber. Rats face retractable levers to the left and right separated by a food hopper. Each trial begins when one of the levers projects into the chamber. The rat presses the lever which is then withdrawn. In order to initiate the second part of the trial, the rat is required to nose poke in the central hopper and this response triggers the entry of both

levers into the chamber. The trial is completed successfully if the original sample lever is pressed again. The order of right and left trials is random and the delay between the two parts of the trials can be varied. This is an example of working memory where sample information must be held and retrieved in order to complete the second part of each trial.

An equivalent task for rhesus monkeys was developed using touch screen technology. The monkey is trained with a 3 x 3 array of lighted panels. On the sample part of the each trial one of the panels is lit and the animal presses it. After a delay during which the screen is blank, all the panels are lit and the trial is successfully completed if the sample position is again touched. The intra-trial delay and the position of the correct panel is varied.

4. Eight arm maze and Morris Watermaze

Spatial working memory is commonly evaluated in the 8-arm maze or in the swim maze. In the former eight arms radiate symmetrically from a central chamber. Food is found at the end of each arm and when trained, rats complete a trial by systematically visiting each arm to retrieve the food. Successful performance must depend on spatial working memory enabling the rat not to re-visit arms from which food has been retrieved. In the swim maze rats are trained to swim in a large pool of opaque water to find and climb onto a platform hidden below the surface. The rat starts each trial from a different point on the circumference of the tank and cannot therefore find the platform by simply swimming in the same direction. Successful performance depends on spatial reference memory for the platform location.

Performance on these tasks is impaired by the cholinergic antagonist, scopolamine, surgical lesions to the hippocampal formation, excitotoxin lesions of the basal forebrain and by a number of other neuropharmacological and surgical manipulations of the forebrain circuits, including the normal ageing process in rodents.

The ability of muscarinic agonists to reverse the behavioural effects of scopolamine

Non-selective muscarinic agonists acting at M₁, M₂ and M₃ receptors reverse scopolamine-induced impairments on long term memory tasks. However, the side effect profile of these compounds also results in general behavioural disruption at the doses which enhance cognition (7).

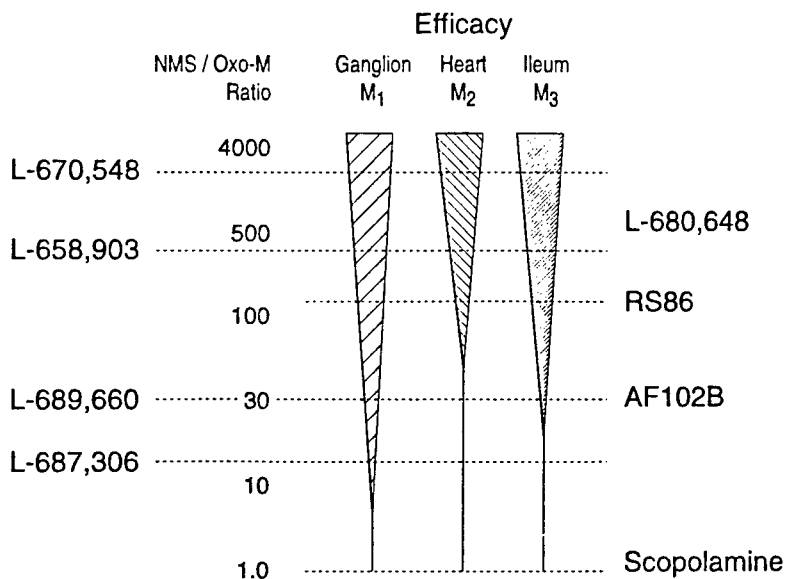
Cholinergic agonists have been sought with selectivity for the M₁ receptors which are considered of most significance for CNS function. Attempts have focussed on several different pharmacological strategies.

(i) lower efficacy at muscarinic receptors or

(ii) selectivity at the M₁ receptor (predominant in brain)

or (iii) functional selectivity achieved by agonist activity at M₁ receptors and antagonist activity at M₂ receptors (reduced cardiac side effects). A number of these compounds also have partial agonist or antagonist activity at M₃ receptors.

A novel series of muscarinic agonists were synthesised (8) in which the ester moiety of arecoline was replaced by an oxadiazole or a pyrazine group. L-670, 548 and L-680, 648 are representative of these compounds; both are potent, non-selective muscarinic agonists with good CNS penetration and profound side effects including salivation, hypothermia, piloerection, tremor and cardiovascular side-effects. Subsequently, in the pyrazine series L-689, 660 and L-687, 306 were identified. These compounds did not exhibit muscarinic receptor selectivity in binding assays but were characterised as partial agonists, exhibiting functional selectivity in



Relationship between the NMS/Oxo-M ratio and functional activity in the three pharmacological preparations. The width of the bar for each tissue represents the relative agonist activity of compounds with a particular NMS/Oxo-M ratio, for each particular functional assay.

Fig. 1

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pharmacological assays with limited receptor reserve (Figure 1). L-689, 660 retained full agonist activity compared with muscarine at the M₁ receptors in rat ganglion, was a potent agonist (but not fully efficacious) at M₃ receptors and acted as a competitive antagonist in the atrium. L-689, 306 was lower down the efficacy scale and yet more selective being a competitive antagonist at both M₁ and M₃ receptors. In this series the side effect profile was less and in the case of L-689, 306 peripheral muscarinic stimulation was not observed at doses of 30 mg/kg.

The oxadiazole and pyrazine derivatives were evaluated for (i) cognitive enhancing properties (ii) general behavioural disruption and (iii) cholinergic side effects in rodents and monkeys.

The pharmacology of muscarinic cognitive enhancers

Muscarinic agonists with functional selectivity at the M₁/M₃ receptors reverse the effects of scopolamine in the rat on passive avoidance, conditioned suppression of drinking test and on delayed matching to position. Independent measures of behavioural performance have been obtained with the response sensitivity task in which rats are trained to press a lever or pull a chain at a high rate sustained by a random interval schedule of reinforcement. This high sustained rate of responding is very sensitive to disruption by non-specific side effects of drugs. It has been possible to demonstrate that functionally selective muscarinic agonists reverse scopolamine effects on cognitive tasks at doses below those which result in general behavioural disruption (9, 10). The window between cognitive enhancing doses and those inducing side effects is not large. Although it is better than that seen with non-selective full agonists, further improvement in this dose ratio needs to be sought in novel muscarinic agonists. Our experience with this range of compounds in monkeys working on the delayed spatial matching task is similar (11).

Acetylcholinesterase inhibitors, which are assumed to stimulate all muscarinic receptor subtypes are surprisingly efficacious in scopolamine models in rats (9, 12), but particularly so in monkeys (13). Physostigmine and its long acting analogue eptastigmine reverse, almost totally, the scopolamine-induced impairment on delayed spatial matching in the monkey. More information on the clinical efficacy of the new generation of acetylcholinesterase inhibitors in AD is keenly awaited. The cognitive enhancing effects of E2020 in rats and monkeys in the absence of side effects and the improved side effect profile for this compound recently reported in man are reasons for encouragement.

Reflections on the behavioural evaluation of muscarinic agonists

Scopolamine remains a valuable pharmacological model for evaluating cognition enhancers provided the following factors are considered:

(i) Scopolamine is a non-selective muscarinic antagonist and therefore will impair a number of specific behavioural functions unrelated to memory. The failure of an agonist to reverse the effects of scopolamine could reflect an inability to normalise these other aspects of behaviour. Equally agonist-induced non-specific side effects could mask the cognitive enhancing property of a drug.

(ii) If a cholinergic agonist reverses the effect of scopolamine on a memory task, it cannot be assumed that the effect is on memory processes rather than some other aspect of higher function. Indeed, it has been shown in the monkey that physostigmine totally reverses the delay related spatial memory deficit induced by scopolamine. However, impairments on this task can be observed in normal monkeys if task difficulty is increased by irrelevant distraction during the delay or extending the delay intervals. Aged monkeys also show a decline in performance. These manipulations would be expected to increase the demands on memory function, yet physostigmine does not reliably improve performance under these circumstances (14).

(iii) Scopolamine deficits can be reversed with non-cholinergic drugs, making the model of general value in the quest for cognitive enhancers. Jensen et al (15), for example, used PA and a sensitive signal detection task to demonstrate that inverse agonists acting at the benzodiazepine receptor were able to reverse the detrimental effects of scopolamine on performance. In monkeys performing a delayed spatial matching task, D-cycloserine was shown to reverse not only the impairment induced by drugs acting at the NMDA receptor but also the effect of scopolamine (16).

(iv) It would be valuable to have behavioural models for evaluating cognitive enhancers which do not depend on the ability of an agonist to reverse an antagonist drug. Memory declines with age, is impaired by lesions to the hippocampal formation, to the frontal cortex and possibly to sectors of the basal forebrain system. These non-pharmacological lesion models should be more fully exploited. An alternative model depends on reducing normal levels of performance by manipulating task difficulty. Monkeys can be trained to show remarkable levels of episodic memory for lists of object stimuli (13). If their recognition memory is stretched by increasing the length of the list of objects to be remembered, cholinergic agonist drugs can significantly enhance performance. But in addition non-selective potent muscarinic agonists, such as arecoline and pilocarpine, and nicotine enhance sub-maximal performance on this task. It may be easier to detect agonist effects in paradigms where the drug does not need to displace an antagonist from the receptor. Indeed, lower doses of agonists were active on this test compared with the reversal of scopolamine on Delayed Matching to Spatial Position. The effects of acetylcholinesterase inhibitors, including E2020, are particularly significant in this model.

(v) The pharmacological and lesion models used are acute manipulations of the brain. The disease being modelled involves chronic and progressive degeneration. Transgenic technology may eventually enable us to induce a neuropathological model which more closely resembles AD.

The cholinergic hypothesis of AD - where now?

The cholinergic hypothesis should remain a driving force in attempts to treat this form of degenerative disease. But there are broader implications when considering the disease and the concept of cholinergic replacement therapy. AD is not a selective cholinergic deficiency disease. Leaving aside the elusive relationship between cholinergic neurone degeneration and plaque and tangle pathology, other neurotransmitter systems are involved (17) and provide targets for drug treatment.

AD is not a homogeneous disease. We should recognise that there are probably subgroups of patients with particular profiles of neurochemical and neurodegenerative loss. This makes it particularly challenging to select appropriate patient groups for drug trials. Structural functional imaging studies may prove valuable in identifying more homogeneous groups of patients for neuropsychological evaluation and drug treatment.

While animal models will remain essential to determine brain penetration of a drug, its duration of action, pharmacokinetics, pharmacodynamics and warn of side effects, they may play a decreasing role in the evaluation of the cognitive effects of drugs. Ultimately novel chemical entities will be evaluated for their ability to activate the memory circuits of the human brain. Measures of *in vivo* pharmacology will be correlated with cognitive performance in the same way that dopaminergic function can now be correlated with motor performance in Parkinsonian patients.

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CLOSING SUMMARY

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The primary emphasis of this symposium was on ligands that are specific for each of the five muscarinic receptor subtypes, their pharmacological properties and possible therapeutic applications. Progress also continues to be made in understanding the nature of the interactions between ligands, receptors and signalling pathways, and in the regulation of receptor function. Particularly striking contributions concerned the structures and properties of a group of structurally related toxins from mamba venoms by Adem, Karlsson, Potter and others, the development by Nathanson and his colleagues of knockout mice lacking the M_1 receptor, reports by several investigators of effects on growth that are mediated by muscarinic receptors, and development of new selective compounds for clinical evaluation.

The M_1 receptor subtype

There can be no doubt that a major impetus for research on muscarinic receptor subtypes has been the possibility that it may lead to a treatment for Alzheimer's disease. Until recently it appeared that even if a sufficiently selective drug were found, it would be only palliative, and would act by replacing the cholinergic tone normally exerted by ascending projections to the cortex that have been shown to degenerate in Alzheimer's disease. The search has been discouraging, although acetylcholinesterase inhibitors may be of modest value. There is now some experimental reason to believe that muscarinic agonists may have not only a palliative effect but may also slow the progress of the disease by altering the mechanisms responsible for the disposal of amyloid precursor protein (APP). Growdon reviewed the rationale for cholinergic enhancement strategies in Alzheimer's disease and the drug trials designed to evaluate this approach, concluding that a selective M_1 agonist should be superior to non-specific agonists or acetylcholinesterase inhibitors because of fewer side effects. Growdon and Nitsch both described effects of muscarinic agonists on amyloid metabolism. Amyloid formation in the brain occurs early in the progress of Alzheimer's disease, and is generally believed to be a major factor in its pathogenesis. The deposits consist primarily of 40-43 residue peptides termed $A\beta$, which are derived by proteolytic cleavage from a transmembrane glycoprotein family known as amyloid precursor protein (APP). The $A\beta$ amyloid fragment has been shown to be neurotoxic. Cleavage within the $A\beta$ domain by α -secretase is followed by the release of the soluble APP ectodomain (APPs), which has trophic and neuroprotective properties. Nitsch reported studies on superfused rat hippocampal and striatal slices and clonal U-373 astrocytoma cells stably transfected with $m1$ receptors and cotransfected with human APP751. These experiments showed that the $m1$ -selective agonist talsaclidine (WAL 2014; (R)-3-(2-propynyloxy)-1-azabicyclo[2.2.2]octane) increased APPs release in a dose-dependent manner and that this increase was blocked by atropine. While further animal studies are required to determine whether talsaclidine and other M_1 agonists can

reduce brain amyloid formation, the authors suggested that talsaclidine may be a useful candidate drug to modulate APP processing and slow disease progression in Alzheimer's disease.

Walland presented data on the effects of talsaclidine on anesthetized dogs. Intravenous infusion ($1 \text{ mg} \cdot \text{kg}^{-1} \cdot \text{min}^{-1}$) produced an increase in plasma catecholamines, increased renal vascular resistance and a biphasic effect on blood pressure, an initial hypotensive phase being followed by a longer phase of hypertension. The effects on renal vascular resistance and blood pressure were blocked by the M_1 antagonist pirenzepine. The authors concluded that talsaclidine is a powerful activator of the sympathetic nervous system and adrenal medulla as a consequence of its actions on M_1 receptors.

Shannon described some of the pharmacological properties of another aceclidine analog, LY297802 ((+)-3-(S)-3-[4-butylthio-1,2,5-thiadiazol-3-yl]-1-azabicyclo[2.2.2]octane), which has been selected for its potential utility in the treatment of pain. Antinociceptive actions are a classical property of muscarinic agents that has been discussed in previous meetings of this series. LY297802 is effective in mouse grid-shock, writhing, tail-flick and hot plate tests, with an oral ED_{50} about 6-fold less than that of morphine. The oral ED_{50} for salivation and tremor was 50-100-fold larger than for analgesia. The antinociceptive effects are competitively antagonized by scopolamine but not by methscopolamine, confirming its central site of action. LY297802 has agonist properties at M_1 receptors but is an antagonist at M_2 and M_3 receptors. It was concluded from these and other results that antinociception is mediated by M_4 receptors. LY297802 is now undergoing clinical development as a novel analgesic.

The prostate gland from several species has been found to contain muscarinic receptor subtypes, but Luthin reported that only the human prostate expresses significant levels of the $m1$ subtype. He described studies on tissues obtained at surgery on cases of benign prostatic hypertrophy, in which the $m1$ receptor was the only detectable subtype, and on PC3, DU145 and LNCaP cell lines derived from human prostate cancer cells, expressing $m3$ (PC3) or an approximately equal mixture of $m1$ and $m3$ subtypes (DU145 and LNCaP). Only PC3 cells responded to carbachol with an increase in PI turnover, and none responded with effects on cAMP metabolism. However, carbachol promoted cell proliferation in all three cell lines. The authors are now investigating the evidently complex relationships between receptor activation and signaling pathways thought to mediate cell division.

The involvement of muscarinic receptors in growth regulation was also addressed later in the symposium by Gutkind and by Joan Heller Brown.

Mamba toxins and Muscarinic receptors

Toxins found in venoms from snakes and other species have played an important role in characterizing receptors and investigating their distribution, but until recently, toxins specific for muscarinic receptors were not known. A workshop was organized to discuss progress in this field, and Adem and Karlsson presented a separate paper on the topic later in the symposium. Eight toxins have been isolated from the venom of the green mamba (*Dendroaspis augusticeps*) and three from the venom of the black mamba (*Dendroaspis polylepis*). Nine toxins have been sequenced, which contain 64-66 aminoacid residues with four disulfide linkages constraining the structure in four loops. This structure is also seen in a number of other snake toxins, and several of the aminoacids are highly conserved. Potter, Jerusalinsky and Harvey summarized their research on these extremely promising tools, investigation of which is presently limited by their very restricted availability. Individual toxins vary in their selectivity among muscarinic receptor subtypes, in their potency and in other

characteristics; most behave as antagonists, but some are allosteric and others competitive; most were reversible but at least one (m1 toxin) was both allosteric and irreversible. Minor differences in sequence can be associated with drastic differences in pharmacological properties.

Later in the symposium Flynn described the use of crude green mamba venom to develop a strategy for the selective labelling of m5 receptors. [^3H]-NMS was used for labelling in the presence of $30\text{ }\mu\text{g}\cdot\text{ml}^{-1}$ venom and $1\text{ }\mu\text{M}$ AQ-RA 741 to occlude most of the m1, m2, m3 and m4 subtypes. Using this approach, the distribution profile of the m5 receptor was found to be distinct from that of the other subtypes, and is enriched in the outer layers of the cortex, specific subfields of the hippocampus, caudate putamen, olfactory tubercle and nucleus accumbens.

There seems little doubt that this group of venom-derived toxins will be of great value in extending our present knowledge of the distribution and functions of the muscarinic receptor subtypes.

The M_2 receptor subtype

In addition to tissue specific functions performed by fully differentiated cells, M_1 and M_2 receptors have been implicated in growth stimulation. Gutkind described research in his laboratory in which expression of M_1 and M_2 receptors has been used as a model for studying proliferative signaling through G protein-coupled receptors. This work leads to the conclusion that mammalian cells have independent signaling pathways connecting cell surface receptors with each member of the MAP kinase superfamily. He presented evidence that $\beta\gamma$ subunits of heterotrimeric G proteins play a role in communicating between M_1 and M_2 receptors and the MAP kinase and the JNK pathways, acting through Ras and Rac1 routes.

Wess discussed the basis of selective receptor coupling with G proteins using muscarinic receptors as model systems. It has previously been shown that individual aminoacids in the second intracellular loop and at the N- and C- terminus of the third intracellular loop dictate selective recognition of Gq/11 proteins by m3 receptors. Using a novel experimental strategy involving the coexpression of hybrid m2/m3 receptors with hybrid $G\alpha$ -subunits, the C-terminus of G-protein $\alpha i/o$ -subunits was shown to be recognized by a short sequence element in the m2 muscarinic receptor that is located at the junction between the sixth transmembrane domain and the i3 loop. This interaction is critically involved in determining coupling selectivity and triggering G-protein activation.

Barnes has investigated homologous and heterologous regulation of M_2 receptor protein and gene expression in human embryonic lung fibroblasts (HEL 299 cells), which constitutively express m2 receptors with no evidence of other muscarinic receptor subtypes. Functional studies on human airways confirmed that prejunctional M_2 receptors inhibit the release of acetylcholine, and may be dysfunctional in patients with asthma, contributing to exaggerated cholinergic reflex bronchoconstriction. The regulation of these receptors thus has a direct bearing on the pathophysiology of asthma. M_2 receptors in HEL 299 cells can be down-regulated by muscarinic and β_2 -adrenergic agonists, by transforming growth factor $\beta 1$, by tumor necrosis factor α and by interleukin 1β , which are elevated in asthma. The mechanisms involved in each case were discussed.

Sheu addressed the identity and function of muscarinic receptors in the heart, which is still in debate. Using the reverse transcriptase-polymerase chain reaction to amplify mRNA from adult rat myocytes, these cells were found to express m1 and m2 receptors. Immunocytochemical analysis confirmed the presence of m1 and m2, but not m3 receptor proteins on the surface of these cells. Evidence was adduced that activation of m1 receptors is involved in the stimulatory effects of muscarinic agonists in mammalian heart.

Levey reported beautiful studies from his laboratory on the distribution of the m2 receptor using double-labelling immunocytochemistry at the electron microscopic level with m2 antibodies and antibodies to the vesicular acetylcholine transporter (VACHT), a specific marker of cholinergic terminals. The results reveal a variety of synaptic localizations of the m2 receptor protein. Localization within VACHT immunoreactive terminals implies a role as a presynaptic autoreceptor; its localisation within non-cholinergic terminals in the hippocampus suggests a role as heteroreceptor in regulating the major excitatory and inhibitory neurotransmitters. Finally, the m2 receptor is found postsynaptically in cells of the basal forebrain and in interneurons in the hippocampus and striatum. These studies clearly indicate that the m2 receptor is not confined to a role as a presynaptic cholinergic autoreceptor.

The M₃ receptor subtype

Nahorski reviewed current ideas about the coupling and regulation of m3 receptors to G proteins. Although m3 receptors couple predominantly to phosphoinositidase C β via Gq/11 proteins, these and other related receptors are promiscuous and can form interactions with multiple G proteins and effectors. Agonist potency therefore depends not only on receptor density but also on the amount and type of G protein that is available in a particular tissue. It is currently uncertain whether all agonists can activate multiple G proteins, or whether different agonists can direct receptors to different transducing proteins. The latter would imply that the concentration and intrinsic efficacy of an agonist might influence the character of a response as well as its intensity. In another revision of classical receptor theory, it is possible that many G protein linked receptors exhibit spontaneous activity in the absence of an agonist, and that receptors exist in both active and inactive forms in the basal state. Agonists, inverse agonists and antagonists would then simply shift the equilibrium between these forms. Finally, the author reviewed studies in his laboratory on the depletion of lipid substrates as a mechanism of receptor regulation.

Allosteric modulators of muscarinic receptors have long been known, and Birdsall discussed the possibility of using differential allosteric modulation of muscarinic receptor subtypes as a means of achieving subtype specificity. He reported that brucine is an allosteric enhancer at m1 receptors, increasing the potency of acetylcholine 2-3 fold, but showed negative cooperativity at m2-m5 receptors. Several analogs of brucine also had allosteric effects, but the subtype specificity differed; N-chloromethylbrucine enhanced the binding and membrane function of acetylcholine 3-5 fold at m3 receptors but was negatively cooperative at m1, m2 and m5 receptors and neutral at m4. It was suggested that a more potent allosteric muscarinic enhancer with appropriate subtype selectivity might be useful in the treatment of Alzheimer's disease.

Zaagsma reviewed the control of airway function by muscarinic receptors. M₁ receptors are localized to parasympathetic ganglia, where they facilitate neurotransmission, and in alveolar walls, where their function is unknown. M₂ and perhaps M₄ receptors occur prejunctionally, and regulate acetylcholine release, and both M₂ and M₃ subtypes are found in smooth muscle, where the M₃ receptors play a major role in producing contraction. In a guinea pig model of allergic asthma, M₂ receptors are dysfunctional, perhaps because of the release of polycationic proteins from eosinophils, leading to bronchial hyperreactivity. A deficiency of endogenous nitric oxide may also contribute to an enhanced postjunctional M₃-mediated response. In airway preparations from both man and experimental animals, this enhanced response was relatively resistant to β -adrenoceptor mediated relaxation. It was suggested that this reduction may be due to transductional crosstalk between phosphatidylinositol metabolism and adenylyl cyclase, which although normally of minor functional importance could be enhanced in allergic asthma.

examined the effects of cholinergic ligands on its reactivity to afferent stimulation. Two new actions of acetylcholine were described: a fast onset short-lived increase in cellular responses to activation of the N-methyl-D-aspartate receptor, and a slow onset, long lasting increase in reactivity to afferent stimulation. The latter effect, which is similar to the familiar long term potentiation (LTP), was named LTPm by the author, and is probably mediated by a postsynaptic M_2 receptor, as indicated by its blockade by methoctramine or AFDX-116 but not by M_1 antagonists. For these and other reasons, the authors propose that LTPm may be related to learning and memory; further support for this proposal comes from their finding that slices taken from aged rats lack LTPm.

Nathanson reported progress in several lines of investigation in his research on the regulation of expression and function of muscarinic receptors. In cultured chick cardiac cells, treatment with carbachol decreased levels of mRNA encoding $m2$ and $m4$ receptors. Activation of mAChR causes both inhibition of the adenylyl cyclase and stimulation of phospholipase C activity; a combination of biochemical and pharmacological approaches was used to demonstrate that both these responses were required for maximal decreases of $cm2$ and $cm4$ mRNA levels. The rate of mRNA degradation was unchanged, suggesting that the rate of gene transcription was reduced in response to carbachol. Treatment *in ovo* also decreased expression of the inwardly rectifying ion channel which is controlled by the receptor. Finally, Nathanson described the use of targeted gene disruption by homologous recombination to generate strains of mice deficient in the $m1$ receptor by deletion of the sequence encoding the amino terminal portion of the $m1$ receptor. This opens the way to elucidating the role of individual receptor subtypes in the mediation of responses *in vivo* by muscarinic receptors.

Three papers in the last session were concerned with muscarinic mechanisms in specific tissues: Fischmeister reviewed muscarinic regulation of the L-type Ca^{++} current (I_{Ca}) in isolated cardiac myocytes; Bolton described the effects of M_2 and M_3 antagonists on currents induced by carbachol in guinea pig ileum; and David Brown reviewed some muscarinic mechanisms in nerve cells.

I_{Ca} is a major determinant of the plateau phase of the cardiac action potential and triggers contraction in cardiac myocytes. β -adrenergic agonists stimulate I_{Ca} in all types of myocytes studied so far by activation of adenylyl cyclase. The increase in cytoplasmic cAMP level activates the cAMP-dependent protein kinase, which is ultimately responsible for the increase in I_{Ca} . Muscarinic agonists reduce the β -adrenergic stimulation of I_{Ca} , which is probably due to the inhibition of adenylyl cyclase. However, evidence has recently been adduced that other mechanisms may be involved, particularly the activation of phosphatase and activation of a pathway involving nitric oxide and cGMP. Resolution of these questions may lie in species differences and/or cardiac tissue differences.

Bolton described experiments on single smooth muscle cells from the guinea pig ileum under voltage clamp. Carbachol evokes a dose-related cationic current which normally would cause depolarization and contraction. This response was antagonized competitively by M_2 antagonists (himbacine, tripitramine and methoctramine) with affinities corresponding to those found in binding studies. In contrast, M_3 antagonists (zamifenacin, HHSD and 4-DAMP) reduced the maximum response to carbachol non-competitively. Further work will be required to distinguish among the alternative explanations for these data.

David Brown provided a succinct review of the neuronal ion currents controlled by muscarinic receptor subtypes and the transduction mechanisms involved. Excitatory effects may result from closure of one or more of a number of K^+ channels, while inhibitory effects generally result from opening of K^+ channels or closure of voltage-gated Ca^{++} channels.

Alabaster reported the discovery and development of two selective M_3 antagonists for clinical use. Revatropate is an M_3 antagonist that shows 50-fold selectivity for M_1 and M_3 receptors relative to M_2 receptors in guinea pig trachea and rat deferens relative to M_2 receptors in atria. This selectivity was also seen in anesthetized guinea pigs and conscious dogs, in which bronchodilator activity was seen without tachycardia. Bronchoconstrictor responses to vagal nerve stimulation were not potentiated by revatropate, which also confirms its lack of effect on M_2 receptors. Revatropate shows promise as an inhaled bronchodilator in patients with chronic obstructive airway disease. Darifenacin is an antagonist that is selective for M_3 receptors relative to both M_1 and M_2 subtypes, and was reported to discriminate between M_3 receptors in different tissues. It inhibits responses of the gut and bladder to cholinergic stimulation without affecting heart rate, and the salivary gland was also resistant to blockade. This agent is undergoing clinical evaluation in the treatment of urge incontinence and functional bowel disease.

Later in the symposium, Nilvebrant reported the pharmacological properties and some clinical data on tolteridine, another agent that is under development for the treatment of urge incontinence and other symptoms of unstable bladder. Unlike the subtype-specific compounds discussed above, tolteridine and its active metabolite, 5-hydroxymethyl tolteridine (PNU-200577) do not discriminate among muscarinic receptor subtypes, but are nevertheless reported to be selective for their effects on the bladder relative to the salivary glands, both experimentally (response to chorda stimulation in anesthetized cat) and clinically, in contrast to darifenacin and oxybutynin, which are more potent in blocking salivation.

Comparative aspects and functional interactions

Joan Heller Brown returned to the question of muscarinic receptor-mediated growth regulation, addressing specifically the question of what determines the ability of muscarinic receptors to induce cell growth. In 1321N1 astrocytoma cells, activation of $m3$ receptors stimulated phospholipase C but does not induce DNA synthesis. In contrast, the thrombin receptor, which also couples to phospholipase C, is strongly mitogenic and induces AP-1-dependent gene expression. This discrepancy is probably not due to receptor desensitization; muscarinic receptor number may be limiting. In ventricular myocytes from neonatal rats, hypertrophic cell growth is induced by heterologously expressed $m1$ or $m3$ receptors but not by the endogenous $m2$ receptors. From studies using chimeric receptors it was concluded that hypertrophy requires both phospholipase C activation and additional signals to induce the morphological features of the response. The authors suggested that small proteins of the Rho subfamily, in addition to G_{12} , mediate growth responses to G-protein-coupled receptors.

Continuing the discussion of the Rho family of proteins, Jakobs described his work on mAChR signaling to phospholipases C and D in human embryonic kidney (HEK) cells stably expressing the $m3$ receptor subtype. Evidence for the participation of Rho proteins in $m3$ signalling to these enzymes was derived from two toxins, Clostridium difficile toxin B and Clostridium botulinum C3 exoenzyme, which inactivate Rho proteins specifically but by different mechanisms. In intact cells toxin B was a potent inhibitor of mAChR-stimulated PLC and PLD activities, but the latter were unaltered in the presence of exogenous phosphatidylinositol 4,5-bisphosphate, suggesting that the toxins inhibit stimulation of PLC and PLD by reducing the cellular level of phosphatidylinositol 4,5-bisphosphate, which was in fact found experimentally. It was concluded that $m3$ mAChR signalling to PLC and PLD in HEK cells requires the concerted action of various intracellular components, of which the complex regulation of phosphatidylinositol 4,5-bisphosphate synthesis is perhaps the most important.

Segal described his recent work on the isolated hippocampal slice preparation in which he

The final paper in the symposium was a review of the behavioral effects of muscarinic agonists and antagonists by Susan Iversen, who focused on the role of acetylcholine in forebrain systems involved in learning and memory. These have acquired particular significance in relation to the understanding of Alzheimer's disease and its treatment. The "cholinergic hypothesis" of Alzheimer's disease proposes that the cognitive impairments in Alzheimer's disease are a consequence of forebrain cholinergic loss, and that cholinergic enhancement may provide a useful strategy for treatment. There is a good deal of experimental and clinical evidence supporting the view that cholinergic agonists can reverse the effects of scopolamine on long term memory tasks, and that cholinergic lesions impair performance on such tasks, but the author warned that failure in memory tasks is not necessarily due to a failure of memory storage, and may as easily be seen if the relevant information is not attended to, or perceived, or if the response mechanism is deficient. These factors may be influenced by the side effects of drugs, and may also be encoded non-cholinergically. Moreover there are obvious differences between animal and human cognition. With these reservations, some of the most useful animal models were briefly described and evaluated.

While non-selective muscarinic agonists acting at M_1 , M_2 and M_3 receptors reverse scopolamine-induced impairments in long term memory tasks, their side effect profiles result in general behavioral disruption at doses that enhance cognition. Since M_1 receptors are considered of most significance for cognitive function, cholinergic agonists were sought with M_1 selectivity or functional selectivity based on agonist activity at M_1 receptors and antagonist activity at M_2 receptors. Among those synthesized were analogs of arecoline in which the ester moiety was replaced by an oxadiazole or pyrazine group. Some of these compounds achieved the objectives of being a potent agonist at M_1 receptors but antagonist at M_2 . These functionally selective muscarinic agonists were capable of reversing scopolamine effects on cognitive tasks at doses below those which result in general behavioral disruption. Finally, the author recommended that in selecting behavioral models for drug evaluation, paradigms should be employed that do not depend on the ability of an agonist to reverse an antagonist drug, for example lesions, aging animals and tasks that place greater demands on memory.

Conclusions

At the time of the first symposium in this series there were many who doubted the existence of muscarinic receptor subtypes as discrete molecular entities, and preferred to interpret the available data in terms of a continuous spectrum of receptor characteristics depending, perhaps, on the microenvironment of each receptor. The broad array of evidence now available, from highly specific toxins to new agents available for clinical use, from aminoacid sequences to detailed maps of sites and mechanisms of interaction, leaves little room for doubt on the matter. Much of this evidence has been generated by those contributing to this meeting and to previous symposia in the series. We all owe a vote of thanks to the organizers, the sponsors and most particularly to our indefatigable Symposium Coordinator, Dr. Ruth R. Levine, for their tireless planning, warm hospitality and hard work.

WORKSHOP

THE USE OF MUSCARINIC TOXINS IN THE STUDY OF MUSCARINIC RECEPTORS

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Summary

One of the most interesting recent developments in the pharmacology of muscarinic receptors has been the finding of small proteins in the venoms of mamba snakes that bind with high affinity and selectivity to different subtypes of muscarinic receptors. In the workshop on muscarinic toxins, the practicalities of isolating, characterising and using these toxins as tools in the study of muscarinic receptors were discussed.

Key Words: muscarinic toxins, mamba snakes, muscarinic receptors

Evert Karlsson described the isolation of several muscarinic toxins from venom of the green (*Dendroaspis angusticeps*) and black (*Dendroaspis polylepis*) mambas, using gel filtration, ion-exchange chromatography and HPLC. Each venom contained several different isotoxins, which all had about 60 amino acid residues in a single polypeptide chain cross-linked by four disulphide bonds. The folding pattern of the muscarinic toxins was the same as other "three-fingered toxins", such as the curaremimetic α -neurotoxins and the anticholinesterase fasciculins.

The different muscarinic toxins have different specificities at subtypes of muscarinic receptors. For example, MT1 and MT2 from green mamba have high affinity for m1 and m4 receptors, and much lower affinity for the other subtypes; MT3 binds with high selectivity to m4 receptors; and MT7 binds exclusively to m1 receptors. With the knowledge of the sequences of the toxins, it is possible to relate changes in subtype specificity to small changes in amino acid sequence.

Lincoln Potter described work in his laboratory that led to the isolation and characterisation of m1-toxin and m4-toxin from *Dendroaspis angusticeps*. m1-Toxin (which is an isotxin of MT7) binds allosterically and irreversibly to m1 receptors with very high affinity, and binds with lower affinity and reversibly to m4 receptors. m4-Toxin (which is the same as MT3) is specific in its binding to m4 receptors, where it is a reversible antagonist.

Because of the low amounts of the toxins in the venoms, genes encoding the muscarinic toxins have been cloned, and m1-toxin has been expressed in *E. coli*. Experiments are continuing in order to improve the yield of toxin from the expression system, but functional recombinant m1-toxin has been obtained.

Diana Jerusalinsky described the characterisation of MT1, MT2 and MT3 from *Dendroaspis angusticeps* through competition binding studies with various radioligands and cloned and native receptors in a variety of tissues. The binding of MT1 and MT2 appeared to be largely irreversible, while that of MT3 was reversible. The toxins were specific to muscarinic receptors, although MT1 and MT2 did show a weaker and reversible interaction at α -adrenoceptors.

MT1, MT2 and MT3 were radiolabelled using ^3H -acetic anhydride. Autoradiograms of rat brain slices showed specific binding of the toxins with differences in the regional distribution of the binding sites for the different toxins.

Alan Harvey discussed the possibilities for using muscarinic toxins as tools in functional pharmacological experiments, especially to investigate the physiological role of different subtypes of muscarinic receptors. This was exemplified by use of MT1, MT2 and MT3 to show that the presynaptic muscarinic receptors in the rabbit vas deferens preparation are likely to be of the M_1 subtype. There was also evidence that MT1 and MT2 acted allosterically on M_1 receptors. Little work has been completed using the muscarinic toxins *in vivo*. However, Dr Jerusalinsky and colleagues have demonstrated that intrahippocampal administration of MT1 and MT2 can increase performance in a learning paradigm in rats, opening the way to study the role of muscarinic receptor subtypes in such complex functions as learning and memory.



ABSTRACTS OF POSTER PRESENTATIONS

1

WAL 1014 FU: AFFINITY FOR MUSCARINIC RECEPTORS FROM RAT TISSUES AND FOR HUMAN MUSCARINIC RECEPTORS EXPRESSED IN CHO CELLS AND FUNCTIONAL EFFICACY IN VITRO:

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Degeneration of cholinergic neurons in the brain of Alzheimer patients has led to the cholinergic hypothesis for the treatment of Alzheimer's disease. M1 selective muscarinic agonists appear to be particularly promising as therapeutic agents since they are devoid of major peripheral cholinergic side effects and exhibit a postsynaptic site of action.

We carried out muscarinic receptor binding with WAL 1014 FU and reference compounds in rat tissue membrane preparations (M1, M2 and M3) where WAL 1014 FU showed comparable binding affinities for the M1 receptor subtype in hippocampus ($[^3\text{H}]\text{Pz}$) $K_i = 6.6 \pm 1.1 \mu\text{M}$ and for the M2 receptor subtype in heart ($[^3\text{H}]\text{NMS}$) $K_i = 6.6 \pm 1.4 \mu\text{M}$ but a 5fold weaker affinity for the M3 receptor subtype from lacrimal glands ($[^3\text{H}]\text{NMS}$) $K_i = 29.6 \pm 6.6 \mu\text{M}$. In membrane preparations from CHO cells expressing the five human muscarinic receptor genes the affinity of WAL 1014 FU for the different receptor subtypes has been determined: $K_i = 25.5 \pm 5.4 \mu\text{M}$ (hm1), $7.1 \pm 3.7 \mu\text{M}$ (hm2), $34.0 \pm 11.2 \mu\text{M}$ (hm3), $17.0 \pm 5.2 \mu\text{M}$ (hm4) and $16.8 \pm 8.1 \mu\text{M}$ (hm5), respectively.

Using the cytosensor technology we investigated the functional efficacy of WAL 1014 FU in vitro at hm1, hm2 and hm3 receptor subtypes expressed in CHO cells in comparison to carbachol and MCN-A-343. This technique measures the efflux of protons stimulated by the agonists. In hm1 transfected cells WAL 1014 FU demonstrated a high degree of intrinsic activity (66.4% when compared to carbachol) and a low intrinsic activity (18.5%) in hm3 transfected cells. No stimulation of the proton outflow could be detected in hm2 expressing CHO cells.

Moreover, we found that WAL 1014 FU in a dose dependent fashion stimulated α -secretion of APP (amyloid precursor protein) in human astrocytoma cells expressing hm1 receptors with comparable efficacy to the full agonist carbachol.

2

DEVELOPMENT OF 5-(3-ALKENYL-1,2,4-OXADIAZOL-5-YL)-1,4,5,6-TETRAHYDRO-PYRIMIDINE DERIVATIVES AS SELECTIVE MUSCARINIC AGONISTS FOR THE TREATMENT OF ALZHEIMER'S DISEASE

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Selective m1 agonists might be useful in the treatment of memory deficits associated with a loss of acetylcholine, as found in Alzheimer's disease. In the current study, we synthesized three 5-(3-alkenyl-1,2,4-oxadiazol-5-yl)-1,4,5,6-tetrahydropyrimidines and examined their binding affinities and agonist activities in rat brain and at m1 receptors expressed in A9 L cells. Molecular modeling studies also examined the interaction of these compounds with a nine-amino acid model of the m1 muscarinic receptor.

The vinyl derivative (CDD-0235) inhibited $[^3\text{H}]\text{-QNB}$ binding to rat brain with an IC_{50} value of $4.4 \pm 1.9 \mu\text{M}$ and stimulated PI metabolism in rat cerebral cortex to $99 \pm 15 \%$. The allyl (CDD-0203) and propenyl (CDD-0216) derivatives both inhibited $[^3\text{H}]\text{-(R)-QNB}$ binding ($2.1 \pm 0.41 \mu\text{M}$ and $5.1 \pm 3.8 \mu\text{M}$, respectively), yet did not dramatically stimulate PI metabolism ($14 \pm 4.0 \%$ and $48 \pm 4.3 \%$, respectively). In A9 L cells expressing m1 receptors, carbachol stimulated PI metabolism to $260 \pm 15 \%$ with an EC_{50} value of $5.5 \pm 0.38 \mu\text{M}$. CDD-0235 stimulated PI metabolism to $120 \pm 16 \%$ with an EC_{50} value of $8.0 \pm 0.75 \mu\text{M}$.

Molecular modeling studies revealed interactions for each ligand between the amidine system and Asp105 and the oxadiazole moiety and Thr192 and Asn381 of the m1 receptor. Differences in agonist activity may be due to the relative contributions of hydrogen bonding and van der Waals interactions between the ligand and receptor. The activity of CDD-0235 warrants further evaluation in the search for m1 agonists for the treatment of Alzheimer's disease. This work was supported by NS 01493, NS 31173 and the DeArce Memorial Foundation.

3

DISCOVERY OF A NOVEL, STRUCTURALLY UNIQUE CLASS OF MUSCARINIC AGONISTS

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The discovery of a novel series of potent muscarinic agonists with unique structural features is reported. Compounds with potent binding to M1 receptors were identified by empirical screening of a large compound library; active compounds were screened further in a functional assay for stimulation of phosphatidylinositol (PI) hydrolysis in CHO cells expressing the human m1 receptor. One compound, an amidinoguanidine, showed potent agonist activity. Subsequent synthetic chemistry resulted in a series of very potent muscarinic agonists. Many compounds were discovered which were full agonists at m1 receptors expressed in CHO cells, with ED50's <10 nM for stimulation of PI turnover. Several of these showed greater than 20-fold selectivity for m1 over m2 receptors in functional assays, and some were as much as 100-fold selective for m1 over m2 receptors. Active compounds tended to be full agonists at m1 sites; some were found to be partial agonists at m2, m3 and m5 sites. Most compounds were also very potent at m4 receptors. We have found that many classical muscarinic agonists show very high potency in the m4/CHO cell line, indicating that the m4 receptor is very efficiently coupled to G-proteins in these cells and the potency of agonists at this subtype may be overestimated. Most compounds in this series showed only modest potency (1-10 μ M) in antagonist (3 H-N-methylscopolamine) binding assays and were non-selective across the five subtypes. *In vivo* studies indicated that these compounds stimulate PI hydrolysis in brain following i.c.v. administration. Compounds from this series represent some of the most potent and m1-selective muscarinic agonists yet described.

4

CONFORMATIONALLY CONSTRAINED ANALOGS OF THE MUSCARINIC AGONIST METHYLTHIO-TZTP. SYNTHESIS, RECEPTOR AFFINITY AND ANALGESIC ACTIVITY

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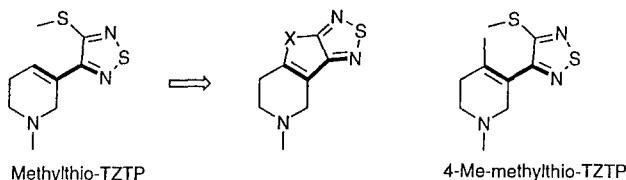
The potent muscarinic receptor agonist 3-(4-methylthio-1,2,5-thiadiazol-3-yl)-1,2,5,6-tetrahydro-1-methylpyridine (methylthio-TZTP) is a relatively flexible molecule. Attempts to predict the active conformation with regard to the angle between the tetrahydropyridine ring and the thiadiazole ring have previously been published (Ward et al. 1992, Periyasamy et al. 1995). The Schulman model suggested the angle to be close to 90° (Ward et al.), whereas the minimum energy conformation predicted the angle to be 180° (Periyasamy et al.).

Tricyclic analogs of methylthio-TZTP, with a fixed angle at 180°, and a rotation hindered 4-Me-methylthio-TZTP analog, with energy minimum close to 90°, have now been synthesized and tested for muscarinic receptor affinity.

A second purpose of synthesizing more rigid analogs of methylthio-TZTP was to improve the analgesic selectivity (Sauerberg et al. 1995).

Consequently, the compounds were also tested for analgesic effects in the mouse grid shock test with simultaneous scoring of the side effects tremor and salivation.

The data showed that the tricyclic analogs had somewhat lower receptor affinity than methylthio-TZTP, but were equally potent as analgesics. The analgesic/side effect ratios had not been improved. The 4-Me-methylthio-TZTP analog was inactive in both tests.



References: Ward et al. J. Med. Chem. 1992, 35, 4011. Periyasamy et al. Brain Research 1995, 693, 118. Sauerberg et al. Life Sci. 1995, 56, 807.

5

STIMULATION OF PHOSPHOINOSITIDE HYDROLYSIS IN VIVO BY XANOMELINE AND OTHER MUSCARINIC AGENTS

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Muscarinic agonists and acetylcholinesterase inhibitors have been used to treat cognitive deficits in Alzheimer's disease. Activation of M1 receptors may stimulate memory processes through activation of phosphoinositide (PI) hydrolysis. The effect of several of these agents in vivo on stimulating PI hydrolysis was compared in mice by prelabeling the phospholipid pool with intraventricularly administered ^3H -myoinositol and measuring conversion to ^3H -inositol monophosphates (IP) in hippocampus after administration of lithium (Patel and Freedman, 1994). The muscarinic agonist-induced stimulation of PI hydrolysis was blocked by centrally acting muscarinic antagonists, demonstrating mediation by muscarinic receptors. The non-selective agonists pilocarpine and oxotremorine increased conversion to IP up to 317 and 193%, respectively. As found in vitro, S-aceclidine more potently increased IP conversion than R-aceclidine. The selective M1 agonist xanomeline (3-30 mg/kg sc) increased conversion of IP in a dose-dependent fashion up to 234 % control. In contrast to the non-selective muscarinic agonists, xanomeline did not produce salivation at doses that stimulated PI hydrolysis. Drug candidates PD142505 (30 mg/kg) and (\pm) SKB20206 (30 mg/kg) increased IP conversion to 106 and 120%, respectively. Acetylcholinesterase inhibitors physostigmine (0.5 mg/kg) and tacrine (30 mg/kg) increased IP to 124 and 119% control, respectively. Thus, xanomeline most effectively stimulates PI hydrolysis in vivo without producing non-selective effects, demonstrating potential for effective therapy in Alzheimer's disease.

6

THE TETRAHYDROPYRIDINE RING CONFERS OPTIMAL M-1 SELECTIVITY AND BLOOD BRAIN BARRIER PENETRATION AMONG HEXYLOXY-1,2,5-THIADIAZOLE AZACYCLES

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Among ester bioisosteres of arecoline that have been investigated as selective m₁ agonists, only the hexyloxy-1,2,5-thiadiazole xanomeline appears to have achieved m₁ functional selectivity. In our present study, the hexyloxy-1,2,5-thiadiazole group of xanomeline was held constant and the tetrahydropyridine was exchanged for other azacycles and azabicycles. The effects of these azacyclic changes on muscarinic affinity, m₁ efficacy, potency, and functional selectivity, were determined. In addition, the effects of these modifications on models of absorption and blood brain barrier penetration as well as pK_a were investigated. Several of the azabicyclic derivatives of xanomeline had comparable if not higher muscarinic affinity, m₁ efficacy, and potency than xanomeline, but also showed higher M₃ activity in vivo. Although the pK_a's of xanomeline and all of its analogs varied by over 1.5 log units, all of the compounds were efficiently absorbed across Caco-2 cells at pH 6 without any major distinction among azacycles being apparent. By contrast, major differences in capacity factors were seen among the various azacycles on immobilized artificial membranes at physiological pH and the capacity factors for xanomeline was uniquely high. This suggests that among the hexyloxy-1,2,5-thiadiazole muscarinic azacycles, the tetrahydropyridine ring of xanomeline confers not only optimal m₁ selectivity but also optimal properties for readily crossing the blood brain barrier

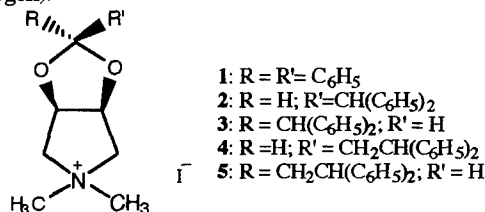
7

ANTIMUSCARINIC ACTIVITY OF BICYCLIC DIOXOLANE DERIVATIVES

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A favored strategy in drug design consists in incorporating the pharmacophore into a rigid structure, often with the aim of determining the bioactive conformation at the receptor surface and giving selectivity in the process of interaction with different receptor subtypes.

Since the antagonist ligands are useful tools for characterizing the different receptor subpopulations (deviating points theory), in our study on muscarinic system we synthesized a series of analogs of 1¹ to test the stereochemistry of the annulation and the optimal distance between the active functions (benzhydryl group and nitrogen).



The synthesized compounds were tested on M₁-M₄ preparations with radioligand binding techniques performed in frozen sections, by displacing the non-selective muscarinic cholinergic receptor antagonist, [³H]-NMS, bound to sections of frontal cortex (M₁), heart (M₂), submaxillary gland (M₃), and striatum (M₄).

Preliminary data indicate that the affinity profile of compounds 4 and 5 is: M₁ ≈ M₄ > M₂ >> M₃.

1) A. Piergentili, P. Angeli, M. Giannella, M. Pignini, W. Quaglia, and S. K. Tayebati, *Arzneim.-Forsch./Drug Res.* **46**, 99-105, (1996).

8

STEREOSELECTIVE INTERACTION OF p-FLUORO-TRIHENYPHENIDYL AND ITS METHIODIDE WITH FOUR MUSCARINIC RECEPTOR SUBTYPES

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The enantiomers of the chiral antiparkinsonian drug, trihexyphenidyl (THP), exhibit high stereoselectivity (up to 400-fold) at native muscarinic receptors, the (R)-isomer being the eutomer with an affinity profile of M₁ (pK_i = 8.9) = M₄ (8.8) > M₃ (8.1) > M₂ (7.7) (Waelbroeck et al., EJP-MPS 227: 33, 1992). In the present study, four analogues with a para-fluoro-phenyl rather than a phenyl group, (R)- and (S)-p-F-THP and their methiodides [(R)- and (S)-p-F-THP⁺], were investigated for their antimuscarinic potency at four muscarinic receptor subtypes. The affinities were determined in functional experiments (pA₂ values) at M₁ (rabbit vas deferens), M₂ (guinea-pig atria) and M₃ receptors (guinea-pig ileum) as well as in radioligand binding studies (pK_i values) at recombinant m1, m3 and m4 receptors expressed in CHO cells and native M₂ receptors present in rat heart.

All compounds acted as pure competitive antagonists in both functional and binding assays. The (R)-enantiomers were more potent (up to 316-fold) and exhibited a greater receptor selectivity than the (S)-isomers. (R)-p-F-THP retained the same affinity profile as the parent compound (R)-THP [M₁ (m1) = m4 > M₃ (m3) > M₂]. N-Methylation changed the receptor selectivity pattern of the tertiary amine (R)-p-F-THP to M₁ (m1) > M₂ = M₃ (m3) = m4

for the methiodide (R)-p-F-THP⁺. This different selectivity profile of (R)-p-F-THP⁺ is based on the fact that N-methylation increased the affinity for M₁ (m1), M₂ and M₃ (m3) receptors up to 16-fold while not affecting the affinity for m4 receptors.

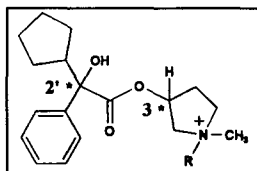
The results show that (R)-p-F-THP⁺ is of special interest for muscarinic receptor classification: it shows qualitatively a similar selectivity profile as pirenzepine (Pfaff et al., Life Sci. 56:1038, 1995), but its affinity for M₁ (m1) receptors is higher (up to 100-fold) than that of pirenzepine.

	M ₁		m1		M ₂		M ₃		m3		m4	
	pA ₂		pK _i		pA ₂	pK _i	pA ₂	pK _i	pA ₂	pK _i	pA ₂	pK _i
(R)-p-F-THP	9.1	9.0	7.6	7.6	8.3	8.2	8.7					
(S)-p-F-THP	6.6	6.8	6.4	6.3	6.3	6.0	6.3					
(R)-p-F-THP ⁺	10.3	9.4	8.7	8.5	8.8	8.8	8.5					
(S)-p-F-THP ⁺	7.8	7.3	7.2	7.0	6.9	6.6	7.2					

9

ANTIMUSCARINIC PROPERTIES OF THE STEREOISOMERS OF GLYCOPYRRONIUM BROMIDE

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Glycopyrronium bromide ($R = CH_3$) is a potent muscarinic antagonist widely used in anesthesia. It contains two centres of chirality, which results in the existence of four stereoisomers. The commercially available drug (a mixture of stereoisomers) has been reported to have high selectivity for muscarinic M1 receptors (Fuder et al., NSAP 347: 591, 1993). The aim of the present study was to examine the affinity of the pure stereoisomers of glycopyrronium bromide (3, 4, 7, 8) at muscarinic receptor subtypes, together with their corresponding tertiary amines (1, 2, 5, 6). Antimuscarinic potency

was determined in functional experiments (pA_2/pIC_{50} values) at muscarinic M1 (rabbit vas deferens), M2 (guinea-pig left atria) and M3 (guinea-pig ileum) receptors as well as in radioligand binding studies (pK_i values) at recombinant m1, m3 and m4 receptors expressed in CHO cells and native M2 receptors present in rat heart.

no.	R	configuration	M1		M2		M3		m3		m4	
			pA_2	pK_i	pA_2	pK_i	pA_2^a	pIC_{50}^b	pK_i		pK_i	
1	H	3S / 2'S	7.70	8.00	7.52	7.40	7.61	6.35	7.93		7.78	
2	H	3S / 2'R	9.98	9.93	9.03	9.10	-	8.80	10.20		9.93	
3	CH ₃	3S / 2'S	8.22	8.36	7.92	7.88	-	6.82	7.82		7.82	
4	CH ₃	3S / 2'R	10.40	10.50	9.39	9.74	-	9.39	10.50		10.30	
5	H	3R / 2'S	8.97	9.08	8.71	8.25	-	8.47	9.18		8.93	
6	H	3R / 2'R	10.00	10.18	9.45	9.22	-	9.05	10.08		9.82	
7	CH ₃	3R / 2'S	9.53	9.36	8.69	9.00	-	8.57	9.63		9.40	
8	CH ₃	3R / 2'R	10.30	10.18	9.43	9.63	-	8.76	10.20		10.27	

^aCompounds 2-8 acted as pseudoirreversible antagonists in the M3 assay. ^bInhibition of neurogenic contractions in guinea-pig ileum.

The results demonstrate that the antimuscarinic potency of compounds 1-8 is highly (up to 1100-fold) controlled by the absolute configuration and the structure of the basic centre of the molecules. However, the pure stereoisomers of glycopyrronium bromide were not found to be M1-selective.

10

PHARMACOLOGICAL CHARACTERIZATION OF PD102807: An m4 SUBTYPE SELECTIVE MUSCARINIC ANTAGONIST.

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The anti-muscarinic agent Artane (trihexyphenidyl) has been used clinically to suppress tremor and relieve rigidity associated with the early stages of Parkinson's disease (PD). However, motor benefits have been accompanied by cognitive dulling. In situ hybridization and antibody localization studies have found that the m4 subtype of muscarinic receptors predominates over m1 in the striatum, while the reverse is true for the hippocampus and cerebral cortex. Thus, an antagonist specific for the m4 receptor subtype over the m1 subtype could be efficacious against PD motor symptoms without deleterious cognitive side effects.

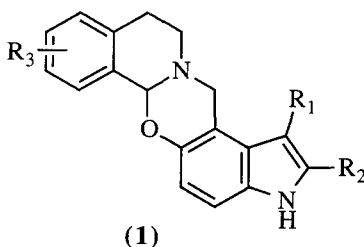
In the search for selective antagonists, membrane preparations from CHO cell lines transfected with each of the human muscarinic subtypes were utilized in [³H]-NMS competition binding experiments. The compound PD102807 was found to have m4 ($IC_{50}=90.7nM$) selectivity of 72-fold against m1 ($IC_{50}=6569nM$), 38-fold against m2 ($IC_{50}=3440nM$), 10-fold against m3 ($IC_{50}=951nM$), and 82-fold against m5 ($IC_{50}=7412nM$). Artane, on the other hand, although found to have considerable m4 affinity, was most potent at the m1 subtype (IC_{50} values; 1.3nM for m1, 18.6nM for m2, 26.3nM for m3, 6.7nM for m4 and 10.3nM for m5). Measurement of functional activity (reversal of the effects of carbachol on PI hydrolysis for m1, m3, and m5 and cAMP accumulation for m2 and m4 receptors) correlated with this profile, as did an autoradiographic study in rat brain where PD 102807 bound with highest affinity in the striatum. In a murine *in vivo* model, PD102807 inhibited spontaneous locomotor activity after i.c.v. administration, however, it showed little effect following oral dosing. Thus, PD102807 may serve as a prototype for the development of an m4-selective antagonist to treat early PD motor symptoms.

11

IDENTIFICATION AND CHARACTERIZATION OF m4 SELECTIVE MUSCARINIC ANTAGONISTS

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Selective m4 muscarinic antagonists may be useful in the treatment of Parkinson's Disease. A series of benzoxazine isoquinolines (**1**) have been synthesized and characterized as potential m4 selective muscarinic antagonists. The affinity of these compounds for the five human receptor subtypes (m1-m5) was determined by [³H]-NMS binding using membranes from transfected Chinese Hamster Ovarian (CHO) cells. One of the most potent and selective compounds of this series is an analog of (**1**) (R₁=CO₂Et, R₂=CH₃, R₃=9-OMe) with an IC₅₀ value of 90.7 nM at m4 receptors, and 72-fold (m1), 38-fold (m2), 10-fold (m3), and 82-fold (m5) selectivities at the other receptors. Functional data (PI hydrolysis and cAMP accumulation) on this particular analog confirmed its muscarinic antagonist properties. The biological activity and structure-activity relationships (SAR) of this series of compounds (**1**) will be discussed.



12

FUNCTIONAL CHARACTERISATION OF PD102807: A NOVEL M₄-SELECTIVE MUSCARINIC ANTAGONIST

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Results obtained in radioligand binding experiments at recombinant m1-m4 receptors expressed in CHO cells indicate that PD102807 (**PD**) might be a muscarinic antagonist selective for m4 over m1-m3 receptors (C.E. Augelli-Szafran et al., and R.D. Schwarz et al.; this volume). Therefore, the aim of the present study was to characterise the interaction of **PD** with native M₁-M₄ receptors using functional assays.

PD, eliciting no agonist response itself, was investigated for muscarinic antagonism at M₁ receptors in rat duodenum (RD: relaxations to 4-F-PyMcN⁺; Pfaff et al., Life Sci. 56: 1038, 1995), M₂ receptors in guinea-pig atria [GPA: negative inotropic responses to arecaidine propargyl ester (APE)] and rabbit vas deferens (RVD: potentiation of neurogenic contractions to carbachol), M₃ receptors in guinea-pig ileum and trachea (GPI and GPT: contractions to APE) and at M₄ receptors in rabbit anococcygeus muscle [RAM: relaxation to (+)-muscarine in histamine-precontracted tissues; Gross et al., Life Sci. 56: 1038, 1995]. Pirenzepine (**Pz**) was used as reference drug. Antagonist potencies were quantified by Schild analysis

(calculated pA₂ values are given in the table).

The pA₂ values of **PD** derived from functional experiments at native M₁-M₄ receptors were very similar to the binding affinities (pIC₅₀ values) obtained at recombinant m1-m4 receptors. The results demonstrate that **PD** is a muscarinic antagonist exhibiting a clear preference for M₄ (m4) receptors. The selectivity of **PD** for M₄ receptors [M₄ > M₃ (10-fold) > M₂ (3-fold) > M₁] is more pronounced than that of tropicamide (Lazareno and Birdsall, BJP: 109, 1993) and of the recently reported M₄-selective antagonist (S)-BS-7826 [M₄ > M₁ (5-fold) ≥ M₃ (2-fold) ≥ M₂ (2-fold)] (Waelbroeck et al., EJP 303: 221, 1996). In addition, **PD** has a selectivity for M₄ receptors greater than that of **Pz** for M₁ receptors, and thus may be a useful tool in the study of muscarinic receptor heterogeneity.

Subtype: Assay	pA ₂	
	PD	Pz
M ₁ : RD	<5.7 [#]	7.96
M ₂ : GPA	5.76	6.73
M ₂ : RVD	5.63	6.71*
M ₃ : GPI	6.08	6.74
M ₃ : GPT	6.07	6.23°
M ₄ : RAM	7.14	7.16

*Eltze et al., EJP 238: 343, 1993.

°Roffel et al., EJP 250: 267, 1993.

[#]No antagonism up to 2 μM.

Supp. by DFG (Graduiertenkolleg: Arzneimittelentwicklung und -analytik).

13

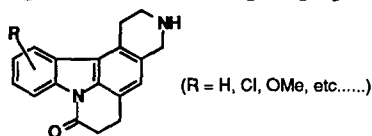
SYNTHESIS OF NOVEL PENTACYCLIC CARBAZOLES AS MUSCARINIC ALLOSTERIC AGENTS.

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The neuromuscular blocker alcuronium (Tucek et al, *Mol. Pharmacol.*, 38:674-680, 1990) and the glycine antagonist strychnine (Lazareno & Birdsall, *Mol. Pharmacol.*, 48:362-378, 1995) are among the few agents reported so far to exhibit positive cooperativity with ³H-NMS at some muscarinic receptor subtypes.

Our SAR studies with strychnine analogues also revealed that brucine (10,11-dimethoxy-strychnine) and some of its N-substituted derivatives (Birdsall, this meeting) could allosterically increase the affinity of acetylcholine. However, these agents bind with low affinities to muscarinic receptors.

In an attempt to increase affinity and to obtain compounds which manifest positive cooperativity with ACh, a series of pentacyclic carbazoles of the general structure shown below, were synthesised. The design of these agents was based on the structure of strychnine, brucine and other structurally related compounds (Gharagozloo et al, manuscript in preparation).



The target molecules were prepared from 3-(tetrahydro-pyridinyl)indoles (Gharagozloo et al, *Tetrahedron*, 10185-10192, 1996) in a multi-step synthesis using an intramolecular Diels-Alder reaction. The binding affinities of these agents as well as their allosteric actions with NMS and ACh will be presented.

14

ALLOSTERIC ACTIONS OF BRUCINE ANALOGS AT MUSCARINIC RECEPTOR SUBTYPES

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It has recently been reported that strychnine acts allosterically at muscarinic receptors (Lazareno & Birdsall, *Mol.Pharmacol.* 48:362-378 1995, Proška & Tuček, *Mol.Pharmacol.* 48:696-702 1995). The allosteric effects of brucine (dimethoxy strychnine) and some of its N-substituted analogs on the binding of ³H-NMS and unlabelled ACh at m1-m4 receptors have now been characterised. We find, using equilibrium and non-equilibrium ³H-NMS binding assays as well as functional ³⁵S-GTPγS binding assays, that brucine analogs enhance both ³H-NMS and ACh affinity in a subtype selective manner. For example, ACh affinity is increased about 2-fold at m1 receptors by brucine, and about 4-fold at m3 receptors by chloromethyl brucine. These results suggest that it may be possible to develop drugs which a) allosterically and selectively increase ACh affinity at muscarinic receptors, and b) show 'absolute selectivity', i.e. positive or negative cooperativity at one subtype and neutral cooperativity at the other subtypes.

15

ALLOSTERIC LIGAND - RECEPTOR - G PROTEIN INTERACTIONS IN LIPID VESICLES

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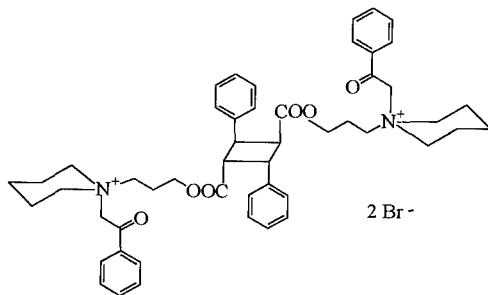
We have found in previous experiments on CHO cells and cardiomyocytes (Jakubík et al., Proc. Nat. Acad. Sci. USA 93:8705-8709, 1996) that ligands binding to the allosteric binding sites of the M₁ - M₄ receptors alter the synthesis of cyclic AMP and of inositol phosphates. Their effects mostly resembled the effects of carbachol but occasionally were in the opposite direction. The data indicated that the allosteric ligands not only influence the binding properties of the classical binding site of muscarinic receptors, but also affect their interaction with the G proteins. In the present study, we wanted to find out if the regulatory action of allosteric ligands on receptor - G protein interaction can also be detected in reconstituted membranes equipped with purified M₂ receptors and Go proteins, and to investigate the importance of the stoichiometric ratio between the receptors and the Go proteins for the observed effects. M₂ receptors purified from the membranes of insect Sf9 cells expressing the human m2 gene, and Go proteins purified from porcine brains were incorporated into liposomes and equilibrated with carbachol, atropine or alcuronium. [³⁵S]GTPγS and GDP were then added and the binding of [³⁵S]GTPγS to liposomes was measured. The rate of [³⁵S]GTPγS binding was accelerated by carbachol and slowed down by atropine. Alcuronium enhanced [³⁵S]GTPγS binding when the receptor:G protein ratio was low and inhibited it when the ratio was high; its 'protean' effects may be explained on the assumption that alcuronium promotes receptor - G protein association but inhibits G protein activation. The effects of atropine testify that the constitutive activity of the M₂ receptors is preserved after their solubilization and incorporation into artificial membranes.

16

TRUXILLIC ACID DERIVATIVES: HIGH AFFINITY, M₂ SELECTIVE ALLOSTERIC MODULATORS. PROBES FOR MAPPING THE MUSCARINIC RECEPTORS.

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The physiological role of each subtype of muscarinic receptor in the central and peripheral nervous systems remains to be clarified, due, in part, to a lack of agonists and antagonists with adequate subtype selectivity. A series of derivatives of α-truxillic acid have been synthesised. The affinity of these compounds for M₁ and M₂ rat muscarinic receptor subtypes was determined by radioligand binding using membranes from cortex and atria. **URA 114** is the high affinity and very effective positive modulator of [³H]NMS binding to M₂ receptors with very low affinity and weak inhibitory effect for M₁ receptors. The biological activity relationships (SAR) of this series of compounds will be discussed, putting special emphasis on the question: which features of the molecules of the allosteric modulators are responsible for their positive allosteric action?



For review: *New Neuromuscular Blocking Agents* (Handbook of Experimental Pharmacology, vol. 79) (Kharkevich, D.A., ed.), 1986, Springer-Verlag

17

ALLOSTERIC INTERACTION ON MUSCARINIC RECEPTORS: FACTS, THEORY AND FANTASIES

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Many aspects of allosteric interaction between ligands on muscarinic receptors have not yet been analysed theoretically. The present contribution provides models and explanation for the time course of the dissociation of a radiolabelled classical ligand induced by a negative allosteric effector at various concentrations, and for the time course of atropine-induced dissociation of the radiolabelled classical ligand in the presence of a high concentration of an allosteric ligand. Explanations of some of the complex binding phenomena described in the literature are provided.

18

STRUCTURALLY CLOSELY RELATED BISQUATERNARY ALLOSTERIC MODULATORS MAY BIND TO DIFFERENT RECOGNITION SITES ON THE M₂-RECEPTOR PROTEIN

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Structure activity relationship investigations with bispyridinium type allosteric modulators suggested that the modulators may be guided into different locations at the M₂-receptor depending on the lateral substituents. WDuo3 carries phthalimidomethyl moieties at both ends of the molecule (WDuo3 = 1,3-bis[4-(phthalimido-methoxyiminomethyl)-pyridinium-1-yl]propane dibromide). Duo3 carries 2,6-dichlorobenzyl moieties instead of phthalimidomethyl. Previous findings showed that the allosteric action of WDuo3 was competitively antagonized by obidoxime. Here, we studied whether obidoxime antagonized the allosteric action of Duo3. The dissociation of [³H]N-methylscopolamine ([³H]NMS) was measured in porcine cardiac membranes (4mM Na₂HPO₄, 1mM KH₂PO₄, pH 7.4, 23°C; control t_{1/2} ≈ 4 min). Duo3 delayed [³H]NMS dissociation concentration-dependently. The apparent rate constant k₋₁ of [³H]NMS dissociation was maximally reduced to < 1 %. The slope factor of the curve for Duo3 amounted to n_H = 2.6. Half-maximum reduction of k₋₁ was achieved at EC_{0.5} = 1.3 μM. In the presence of obidoxime the allosteric action of Duo3 was diminished. Obidoxime concentration-dependently induced a nearly parallel rightward shift of the concentration effect curve of Duo3. The antagonistic effect was analysed nonlinearly according to Lew & Angus [TiPS 16, 328-337, 1995]. The best fit was obtained assuming a non-competitive interaction. The resulting pA₂ value amounted to 3.00 ± 0.08, and n (equivalent to the slope factor in a Schild regression) was 0.51 ± 0.03 (means ± S.E., five concentration effect curves). In comparison, the antagonistic effect of obidoxime against WDuo3 was characterized by a slope factor of unity and a pA₂ of 4.16 ± 0.07.

In conclusion, the divergent sensitivities of Duo3 and WDuo3 towards the antagonistic action of obidoxime may point to different recognition sites on the M₂-receptor protein for these structurally closely related compounds.

19

INFLUENCE OF THE LATERAL SUBSTITUENTS OF BISPYRIDINIUM COMPOUNDS ON THE MODE OF ALLOSTERIC ACTION ON MUSCARINIC M₂-RECEPTORS

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WDuo3 and Duo3 are structurally closely related compounds containing phthalimidomethyl- (PhthM) or 2,6-dichlorobenzyl- (DCB) residues, respectively, at both ends of a bispyridinium middle chain. WDuo3 and Duo3 allosterically retard the dissociation of [³H]N-methylscopolamine ([³H]NMS) from muscarinic M₂-receptors. There are several lines of evidence pointing to different recognition sites on M₂-receptors for these compounds: The concentration-effect-curve (CEC) for the allosteric reduction of k₋₁ of [³H]NMS dissociation is steep in case of Duo3 and normal in case of WDuo3. The dependence of the effect on the ionic composition of the incubation buffer is remarkably smaller with Duo3 than with WDuo3. The sensitivity towards the antagonistic action of obidoxime is different.

Here, we studied the allosteric action of a hybrid compound (4-[[[(phthalimido-methoxy) imino] methyl]-1-[3-[4-[[[(2,6-dichloro-benzoyloxy) imino] methyl] pyridino] propyl] pyridinium dibromide) containing PhthM at one end of the molecule and DCB at the other end. [³H]NMS dissociation was measured in porcine cardiac membranes (4 mM Na₂HPO₄, 1 mM KH₂PO₄, pH 7.4, 23°C; control t_{1/2} ≈ 4.5 min). The hybrid compound retarded [³H]NMS dissociation concentration-dependently. The CEC for the reduction of k₋₁ levelled off at k₋₁=0 and was characterized by an inflection point at EC₅₀=90nM. The slope factor n_H=1.3 was near unity as in case of WDuo3. Also, the influence of the buffer composition on the allosteric potency was similar compared with WDuo3. The allosteric effect of the hybrid was antagonized by obidoxime. Obidoxime was found to shift the CEC to the right by a factor of two at pA₂=4.17. This value corresponds with the pA₂=4.16 of WDuo3, but not with the pA₂=3.00 of Duo3. However, the concentration dependence of the antagonistic effect of obidoxime against the hybrid was flat as found with Duo3, but not with WDuo3.

It is concluded that the PhthM residue may guide the hybrid preferentially into the WDuo3 recognition site. If this site is occupied by obidoxime the hybrid might also utilize the Duo3 recognition site.

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INFLUENCE OF ALLOSTERIC MODULATORS OF MUSCARINIC RECEPTORS ON THE DISSOCIATION CHARACTERISTICS OF RADIOLIGANDS OF A₁-ADENOSINE-, α₁- AND β-ADRENERGIC RECEPTORS.

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In order to demonstrate the specificity of allosteric modulators of the M₂-muscarinic receptor, the dissociation characteristics of ligands of adenosine A₁-, α₁- and β-adrenergic receptors have been studied. For this purpose the effects of three representative allosteric modulators, alcuronium (ALC), W84 (N,N,N',N'-tetramethyl-N,N'-bis-(3-phthalimido-propyl)-N,N'-hexane-1,6,-diyl-bis-ammonium dibromide), and gallamine (GAL) on the equilibrium binding and the dissociation of the adenosine A₁-receptor ligand [³H]-cyclopentyl-dipropylxanthin (CPX), the α₁-adrenoceptor ligand [³H]-prazosin (PRAZ) and the β-adrenoceptor ligand (-)-[¹²⁵I]-iodocyanopindolol (ICYP) have been investigated. The membrane fraction of homogenized rat cardiac left ventricle (ICYP, PRAZ) or rat whole forebrain (CPX) was incubated for 45 min with PRAZ or for one hour with ICYP or CPX. At the end of the incubation period the equilibrium binding or, after the addition of an excess concentration of unlabelled cyclopentyl-dimethylxanthin (A₁, 10 μM), phentolamine (α, 10 μM) or (±)-CGP 12,177 (β, 10 μM) the dissociation was determined in the presence or the absence of the allosteric modulators. All values are given as mean ± SEM of 4-6 observations. CPX showed a monophasic dissociation pattern with a half-life of 6.0 ± 0.3 min in the absence and of 6.0 ± 0.2, 6.5 ± 0.1 and 6.0 ± 0.1 min. in the presence of 30 μM ALC, 30 μM W84 and 1000 μM GAL, respectively. No influence on the CPX-equilibrium binding was detectable. The same dissociation pattern was observed for PRAZ with the respective half-life of 16 ± 1 min in the absence and of 17 ± 1, 8 ± 1 and 13 ± 1 min. In the presence of the allosteric modulators, the PRAZ equilibrium binding amounted to 36 ± 2% (ALC), 93 ± 2% (W84) and 42 ± 2% (GAL) of control. Apparently, ALC and GAL interfere with the association of PRAZ. Since the dissociation of ICYP was slow and after 3 hours more than 40% of the radioligand were still bound, no reliable half-life could be calculated. However, at no time point the binding of ICYP in the absence and the presence of 30 μM W84 showed differences. In the presence of GAL and ALC, the dissociation curve of ICYP revealed an initial offset from the starting level followed by the major phase of dissociation being parallel to the control curve. The equilibrium binding of ICYP amounted to 92 ± 6% (ALC), 83 ± 8% (W84) and 91 ± 5% (GAL) of control. In conclusion, the allosteric alteration of radioligand dissociation by ALC, W84, and GAL does not appear to be a common feature of G-protein coupled receptors.

21

IDENTIFICATION OF RESIDUES CONTRIBUTING TO THE SUBTYPE SELECTIVITIES OF GALLAMINE AND UH-AH 37 AT MUSCARINIC RECEPTORS.

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Gallamine is the prototypical muscarinic allosteric ligand and has a marked preference for the m2 subtype. UH-AH 37 is a competitive ligand related to pirenzepine that has lower affinity at the m2 receptor, compared to the other four subtypes. Nonetheless, the m2/m5 subtype selectivities of both of these ligands have previously been attributed to a short (31 amino acid) segment of the receptor sequence, predicted to make up most of the sixth transmembrane domain and all of the third outer loop of the receptor structure^{1,2}. We have used site-directed mutagenesis to change the amino acid residues in this stretch of the m5 receptor to the corresponding amino acids of the m2 receptor one at a time; in the two cases where there were adjacent residues to be mutated, we also prepared the corresponding double mutation. None of these mutations produced marked changes in the affinity or rate of dissociation of the labeled ligand, [³H]NMS. For UH-AH 37, only one mutation (thr to ala at m5⁴⁵⁶) resulted in a reduction of affinity similar to that seen when the entire segment was substituted. Surprisingly, two mutations led to enhancements of affinity for gallamine that were similar to that seen when the entire segment was substituted. These were lys to pro at m5⁴⁷⁰ and val to asn at m5⁴⁷⁴. However, the double mutant asp-lys to ala-pro at m5⁴⁶⁹⁻⁴⁷⁰ did not enhance the affinity for gallamine, leading us to believe that the enhancement of affinity produced by the single substitution at m5⁴⁷⁰ was due to an anomalous interaction with asp⁴⁶⁹, which is present in the wild-type m5, but not at the corresponding position in m2. Thus, the lower affinity of UH-AH 37 for the m2 receptor appears to be due to the lack of the threonine that is present at m5⁴⁵⁶ (and also at the corresponding location in the m1, m3, and m4 subtypes). On the other hand, the higher affinity of gallamine for the m2 receptor appears to be due to an interaction with the asparagine at m2⁴¹⁹, which does not occur at the corresponding position in m5 or in any of the other subtypes.

¹Wess et al., Mol. Pharmacol. 41:369 ²Ellis et al., Mol. Pharmacol. 44:583

[Supported by R01 AG05214]

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A COMMON SITE OF ACTION FOR TWO ALLOSTERIC ANTAGONISTS.

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Combination of two competitive antagonists produces additive dose ratios when tested with an agonist in functional experiments, whereas the allosteric antagonist heptane-1,7-bis(dimethyl-3'-phthalimidopropyl) ammonium bromide (C₇/3'-phth), in combination with a number of competitive antagonists, produces supra-additive dose ratios (Christopoulos & Mitchelson, 1994). To test whether C₇/3'-phth acts at a common site to the allosteric antagonist gallamine, experiments with combinations of the antagonists were conducted on the negative inotropic response to carbachol in guinea pig atria. Combination of C₇/3'-phth (10 μ M) with the competitive antagonist, atropine (0.2 μ M), produced a dose-ratio which was *ca.* 6-fold greater than expected if both antagonists were acting competitively. The combination of gallamine (100 μ M) with atropine gave a combined dose-ratio which was close to that expected for two competitive antagonists, although with acetylcholine as agonist the combined dose ratio was *ca.* 66% of that produced by atropine alone. Combination of C₇/3'-phth (10 μ M) with gallamine (100 μ M), using carbachol as the agonist, gave a combined dose ratio which was less than that expected for two competitive antagonists. The findings could be explained with a model involving C₇/3'-phth and gallamine competing for a single site with co-operativity factors (α) of *ca.* 1000 and 200 respectively for their interaction with carbachol at the allosteric site; values of a similar order for the co-operativity factors were obtained from separate experiments investigating the effect of each antagonist alone on responses to carbachol.

Christopoulos A. and F. Mitchelson (1994) Mol. Pharmacol., 46, 105-114.

23

ALLOSTERIC EFFECTS OF CATIONS ON MUSCARINIC RECEPTORS.

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We reevaluated the effect of cations on muscarinic receptors recognition. Monovalent cations (Na^+ , Li^+ and K^+) inhibited competitively [^3H]NMS binding to muscarinic receptors, facilitated the receptor recognition by uncharged (carbo) analogues of muscarinic antagonists, and accelerated the dissociation rate of [^3H]NMS. We compared the binding properties of wild type M_1 receptors and of a point-mutated Asn^{71} M_1 receptor. Monovalent cations were competitive inhibitors of [^3H]NMS binding to both cell lines, but did not accelerate [^3H]NMS dissociation from the mutant Asn^{71} M_1 receptor, and increased only 2-fold (instead of 10-fold) the affinity of uncharged compounds for the receptor. Divalent cations (Ca^{++} , Mg^{++} , Mn^{++}) behaved as allosteric antagonists of [^3H]NMS binding to the receptors. They decreased the tracers' affinity and dissociation rate from the receptors, and inhibited competitively the allosteric effect of gallamine on tracer recognition.

Receptor recognition by divalent cations and by gallamine was extremely sensitive to monovalent cations (Hill coefficient close to 2) suggesting that several acidic groups (glutamate or aspartate residues) are probably involved in their interaction with muscarinic receptors. In low ionic strength buffers, divalent cations had high affinities (K_D : 10 - 100 μM) for muscarinic receptors.

24

EVIDENCE FOR THE PRESENCE OF TWO SITES FOR CLASSICAL LIGANDS ON MUSCARINIC RECEPTORS

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It has been proposed repeatedly that muscarinic receptors undergo a slow conformational change (isomerization) after the binding of certain ligands, which explains the unusual features of the association or dissociation of these ligands. We observed several phenomena which seem to be best explained on the assumption that muscarinic antagonists associate with two sites in sequence, presumably moving from a superficially located peripheral site to a more deeply located central site. Under most conditions, only one of the two sites can be occupied by a reversible ligand. Taken together, the following experimental observations (made on membranes of CHO cells expressing the M_1 receptors) seem to be best explained by this mechanism: (1) The dissociation rates of N-methylscopolamine (NMS) and quinuclidinyl benzilate (QNB) are fast after short-lasting prelabelling and slow after long-lasting prelabelling. (2) High concentrations of NMS and QNB slow down the dissociation of [^3H]NMS and [^3H]QNB from the receptors after long-lasting but not after short-lasting prelabelling. (3) Given sufficient time, the irreversible ligand [^3H]prolylbenzylcholine mustard ([^3H]PBCM) labels 80% more sites than do [^3H]NMS or [^3H]QNB. After the membranes had been very mildly treated with benzylcholine mustard (BCM) so that the binding of [^3H]NMS became impossible, [^3H]PBCM could still associate with the membranes and the rate of its association was about 50% diminished by 1 mM NMS or 1 mM carbachol, and about 90% diminished by 1 mM alcuronium. (4) When applied after the receptors had been labelled with [^3H]NMS, BCM prevents the dissociation of [^3H]NMS. - Additional experiments suggested that aspartate in position 99 of M_1 receptors may be an important constituent of the peripheral site, since high concentrations of NMS or QNB were less efficient in inhibiting the dissociation of [^3H]NMS or [^3H]QNB from the D99N mutants than from the wild type M_1 receptors, and BCM did not prevent the dissociation of [^3H]NMS from the D99N mutants.

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A COMPARISON OF THE BINDING PROFILE OF ANTAGONISTS AT THE HUMAN CLONED MUSCARINIC RECEPTORS m_1 AND m_3 USING [3 H]-DARIFENACIN AND [3 H]-NMS

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We have compared the binding of [3 H]-darifenacin with that of the non-selective muscarinic antagonist [3 H]-N-methyl scopolamine (NMS) to m_1 and m_3 receptors. In contrast to [3 H]-NMS, which is non-selective, [3 H]-darifenacin binds with 5 fold higher affinity to m_3 ($K_D = 0.33$ nM) than m_1 ($K_D = 1.6$ nM) and with little, or no, specific binding to m_2 , m_4 and m_5 receptors (Smith and Wallis, 1996). Typically 30-50 μ g of protein was incubated for 2h at 25° C in 20mM Hepes buffer (pH 7.4). Non specific binding was defined by 1 μ M atropine. The pharmacology of standard antagonists at displacing [3 H]-NMS and [3 H]-darifenacin was similar (see table) confirming that [3 H]-darifenacin behaves in a comparable manner to [3 H]-NMS at m_1 and m_3 receptors. Given the novel binding affinity profile of [3 H]-darifenacin (i.e. its m_3 selectivity) these data provide further evidence that this radioligand will be a useful tool with which to study M_3 receptors, especially in tissues expressing multiple muscarinic receptor subtypes.

Compound	[3 H]-Darifenacin m_1	[3 H]-NMS m_1	[3 H]-Darifenacin m_3	[3 H]-NMS m_3
Darifenacin	8.36 \pm 0.24	8.15 \pm 0.09	9.14 \pm 0.12	9.12 \pm 0.22
Atropine	9.53 \pm 0.23	9.55 \pm 0.06	9.40 \pm 0.21	9.56 \pm 0.25
4-DAMP	9.04 \pm 0.07	9.04 \pm 0.13	9.19 \pm 0.20	9.25 \pm 0.17
Methoctramine	7.28 \pm 0.19	6.93 \pm 0.12	6.63 \pm 0.14	6.85 \pm 0.16
Pirenzepine	8.63 \pm 0.13	8.06 \pm 0.10	6.85 \pm 0.08	7.05 \pm 0.20

Smith CM and Wallis RM (1996): J.Receptor and Signal Transduction Research. In Press

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THE INTERACTIONS OF THE ENANTIOMERS OF ACECLIDINE WITH SUBTYPES OF THE MUSCARINIC RECEPTOR

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The pharmacological activity of the enantiomers of aceclidine was investigated in Chinese hamster ovary (CHO) cells transfected with the M_1 through M_5 subtypes of the muscarinic receptor and also in the rat heart and parotid gland which express primarily M_2 and M_3 receptors, respectively. When measured by stimulation of phosphoinositide hydrolysis in CHO cells transfected with the M_1 , M_3 and M_5 subtypes, the potency of S(+)-aceclidine was approximately two- to four-fold greater than that of R(-)-aceclidine, whereas the maximal response of the R(-)-isomer was only 44 to 66% of the S(+)-isomer. When measured by inhibition of forskolin-stimulated cAMP accumulation in CHO cells transfected with the M_2 and M_4 subtypes, the potency of S(+)-aceclidine was approximately 3.5-fold greater than that of R(-)-aceclidine. The activity of the enantiomers of aceclidine at native M_2 and M_3 receptors coupled to inhibition of adenylyl cyclase activity in the heart and stimulation of phosphoinositide hydrolysis in the parotid gland, respectively, were similar to those observed in CHO cells transfected with the corresponding receptor subtypes. We devised a simple quantitative method for using our data in CHO cells to predict the relative potencies of agonists in a more sensitive assay where they produce a full maximum response. Using this method, we were able to predict the relative potencies of the enantiomers for eliciting contractions in the guinea pig ileum, an M_3 response, from their activity in CHO cells transfected with the M_3 subtype. Our method of analysis should have application in a variety of studies where transfected cells are used to determine the pharmacological activity of agonists.

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ASP122 AND TYR124 IN THE M1 MUSCARINIC RECEPTOR ARE CRITICAL FOR RECEPTOR FOLDING BUT NOT FOR SIGNALLING.

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An AspArgTyr triad occurs in a majority of rhodopsin-like G protein-coupled receptors. The fully-conserved Arg is critical for G protein activation, but the function of the flanking residues is not well understood. We have expressed m1 muscarinic receptors, mutated at Asp122 and Tyr124, in COS-7 cells. Most mutations at either position strongly attenuated or prevented the expression of binding sites for the antagonist [³H]N-methylscopolamine. Receptor protein, visualised with a C terminally-directed antibody, was reduced but never completely abolished. The effects of these mutations were partially reversed by the deletion of 129 amino acids from the third intracellular loop of the receptor. In several cases, comparison of immunocytochemistry with binding measurements suggested the presence of substantial amounts of inactive, presumably misfolded, receptor protein. None of the variants which bound [³H]N-methylscopolamine underwent large changes in their affinities for acetylcholine, and all retained nearly normal abilities to mediate an acetylcholine-induced phosphoinositide response. We propose that Asp122 and Tyr124 make intramolecular contacts which are critical for receptor folding, but that they do not participate directly in signalling. The role of these residues is completely distinct from that of Arg123, whose mutation abolishes signalling, but without diminishing receptor expression.

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OVER-EXPRESSION AND PURIFICATION OF ENGINEERED M1 MUSCARINIC RECEPTORS.

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The application of crystallographic and other physical techniques to muscarinic acetylcholine receptors requires the availability of large amounts of pure, active receptor protein. Amongst the factors which limit the utility of the wild-type receptor sequences are susceptibility to proteolysis, and inefficiency of purification techniques.

In an attempt to overcome these deficiencies, we have deleted the greater part of the third intracellular (i3) loop of the m1 mAChR, and introduced a hexa-His tag by replacement of the C-terminus. The resultant construct mediated a robust phosphoinositide signal in COS-7 cells. Expression as a malE-fusion protein gave levels up to 20 pmol mAChR/mg membrane protein in *E. Coli*. Insertion into a Baculovirus vector, which also added an N-terminal mellitin signal sequence and a myc tag, allowed the isolation of a recombinant virus which gave 40 pmol mAChR/mg membrane protein after infection of SF9 cells. These levels were approximately 20 fold greater than those given by the wild-type sequence. In *E. Coli*, labelling with [³H]-propylbenzylcholine mustard showed that the i3 loop deletion greatly improved the resistance of the receptor to proteolysis.

The receptor constructs have been solubilised successfully as [³H]N-methylscopolamine complexes, stabilised by the allosteric modulator strychnine. They have been very extensively purified by immobilised metal ion chromatography, using the C-terminal His-tag. They may provide a convenient source of pharmacologically intact receptor for physical studies.

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CYSTEINE SCANNING MUTAGENESIS OF TRANSMEMBRANE DOMAIN V OF THE M1 MUSCARINIC RECEPTOR.

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The aim of this study was to make positive identifications of amino acids in transmembrane domain (TMD) V of the m1 muscarinic receptor (mAChR) which contact the acetylcholine sidechain. The principal approach used was cysteine-substitution mutagenesis followed by chemical reaction. Consecutive residues (Ile188-Ala196) in TMD V of the rat m1 mAChR were mutated to cysteine, and to alanine or glycine. Mutant receptors were probed by binding of the antagonist [3 H]N-methylscopolamine ([3 H]NMS), the agonist acetylcholine, and the sulfhydryl-alkylating ACh analogue, bromoacetylcholine (BrACh).

Only the mutation of Ala193 affected the binding of [3 H]NMS, causing a 5-fold decrease in affinity. In contrast, ACh affinity was strongly decreased by the mutation of Ile188, Thr189, Thr192, and Ala196, residues which may be modelled as lying on one face of an α -helix. Interestingly, Phe190Cys caused a significant increase in ACh affinity.

The reversible binding of BrACh resembled that of ACh, and was similarly affected by the mutations. In addition, BrACh caused rapid, irreversible blockade of the Thr192Cys mutant, and showed evidence of a lower rate of reaction with Ala193Cys, Ile188Cys, and Ala196Cys, but not with the other Cys mutants, or the wild-type receptor. These observations pinpoint Thr192 as a primary candidate for contact with the acetyl methyl group of ACh, and suggest that a penumbra of surrounding residues, particularly Ala196, may also participate in a putative methyl binding pocket.

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PHENOTYPIC INTERCONVERSION OF MUSCARINIC RECEPTOR SUBTYPES COUPLED TO PHOSPHOLIPASE C BY EXCHANGING TWO AMINO ACIDS.

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Comparison of the m1, m3 and m5 muscarinic acetylcholine receptor (mAChR) sequences reveals that the terminal segments of the third intracellular (i-3) loop are well conserved with the exception of two amino acids. The amino- and carboxyl-terminal segments of the m1 mAChR correspond to the sequences RIYRETENR and LVKEKKAAR (underlined amino acids in the m1 sequence are not conserved in the m3 and m5 sequences), respectively. The KKAAR motif, contained within the LVKEKKAAR segment of the m1 mAChR, is critical for agonist-mediated signaling (Lee et al., Mol. Pharmacol. 50: 140-148, 1996). In both the m3 and m5 mAChRs, the amino- and carboxyl-terminal sequences are RIY(K/R)ETEKR and L(I/V)KEKKAQ, respectively. Carbachol (CBC) increases phosphoinositide (PI) hydrolysis in Chinese hamster ovary (CHO) cells transfected with the m1, m3 or m5 mAChR subtype. The magnitude of this increase is density- and subtype-dependent. Titration of wild-type m3 mAChR densities in CHO cells to ~100 fmol/mg protein yielded no PI response following CBC challenge. In cells expressing wild-type m1 mAChRs at similar densities, CBC elicited a 4-fold increase. To determine if the two non-conserved amino acids in the membrane proximal i-3 loop are responsible for these signaling differences, we have mutated these residues and expressed the mutant receptors in CHO cells. Mutation of the non-conserved amino acids of the m3 mAChR to the corresponding residues in the m1 sequence yielded an m3 mutant with a PI response that phenotypically resembles the wild-type m1 mAChR. Conversely, a wild-type m3 phenotype was displayed when either one of these non-conserved residues in the m1 mAChR was mutated into the corresponding residue found in the m3 sequence.

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MOLECULAR STUDIES OF AGONIST INTERACTIONS WITH WILD-TYPE, T234A, AND N507A m3 RECEPTORS

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Previous site-directed mutagenesis studies have identified several amino acid residues important for binding and activity of ligands at m3 muscarinic receptors.^{1,2} To further clarify the precise nature of ligand interactions with specific amino acid residues, we examined the binding and activity of a series of muscarinic agonists at wild-type and mutant m3 receptors.

Using plasmids obtained from Dr. Wess (NIDDK), wild-type and mutant (T234A and N507A) m3 receptors were expressed stably in CHO-K1 cells. After selection, twenty muscarinic agonists were examined for agonist activity at wild-type and mutant receptors. In general, full muscarinic agonists such as carbachol and oxotremorine-M displayed high efficacy at wild-type m3 receptors, intermediate efficacy at N507A receptors and lowest efficacy at T234A receptors. Agonist potencies were reduced three- to ten-fold at T234A receptors, yet appeared unchanged or even higher at N507A receptors.

Agonist affinity was assessed through the inhibition of [³H]-(R)-QNB binding to each receptor. Carbachol and oxotremorine-M affinities for wild-type, T234A and N507A receptors were in general agreement with agonist potencies at each receptor. In contrast, some ligands, such as the ethyl and propargyl esters of 1,4,5,6-tetrahydropyrimidine, exhibited comparable affinities for wild-type, T234A and N507A receptors. These data point to a discrepancy between agonist binding and potency, and highlight molecular features important for agonist activity and selectivity.

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- (1) Wess, et al. *J. Biol. Chem.* **1992**, 267, 19313-19319.
- (2) Bluml, et al. *J. Biol. Chem.* **1994**, 269, 18870-18876.

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MUSCARINIC ANTAGONISTS METHOCTRAMINE AND HHSiD CAN INHIBITE G-PROTEIN ACTIVITY.

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Acetylcholine released from the vagus nerves causes contraction of the airway smooth muscle through M3 receptors, whereas inhibitory neuronal M2 muscarinic receptors limit acetylcholine release. The M2 receptors are guanine nucleotide-binding protein-coupled receptors which have been shown to exist in two states, a high affinity agonist state and a low affinity state. In this study we were looking forward if some muscarinic ligands could behave as inverse agonists in a pig atrial membrane preparation enriched in muscarinic M2 receptors. Intrinsic activity was assessed by measurement of GTPase activity. Maximal increase was observed with 0.5 mM of carbachol (18.26 pmol Pi/15 min/pmol of recept.). Atropine acts as a neutral antagonist because it didn't modify basal activity. Surprisingly methoctramine induced a negative GTPase activity. At 1 mM, it inhibits basal GTPase activity by 26.49 ± 2.75 pmol Pi/15 min/pmol of receptor below basal activity. This activity was not antagonized by atropine and subsisted after ADP ribosylation with pertussis toxin. Moreover high concentrations of methoctramine inhibits GTPase activity induced by mastoparan (which acts directly on G-protein). The same effects were found for HHSiD, with a maximal inhibition of 42.38 ± 2.99 pmol Pi/15 min/pmol of receptor. As GPant-2 can antagonize the effect of mastoparan we used it to antagonize the effects of each latter muscarinic antagonist. Our observations showed that GPant-2 didn't antagonize the effect of neither methoctramine nor HHSiD.

In conclusion we have identified two muscarinic antagonists which can inhibit GTPase activity. They are not inverse agonists because their actions are not mediated by the receptor. However they are perhaps direct inhibitors of G-proteins by acting on an actually unknown binding site.

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AN IMMUNOBLOT ASSAY FOR PTX ADP-RIBOSYLATION OF BRAIN AND SPINAL CORD Gi/O PROTEINS

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Pertussis toxin (PTX) blocks inhibitory G-proteins (Gi/O) coupled to muscarinic M2 and M4 receptors by ADP-ribosylation, and can be used to indicate participation of these subtypes in mediating physiological responses. PTX ADP-ribosylation of membranes *in vitro* is usually determined using [32 P] NAD as substrate coupled with SDS-PAGE and autoradiography to identify labeled Gi/O. This study describes an alternative method.

Membranes from rat brain and mouse spinal cord were solubilized by sonication in Tris buffer containing 1% sodium cholate. PTX was activated by incubation in 50 mM DTT. The ADP-ribosylation reaction (50 μ l) contained 25 mM Tris-HCl pH 8.0, 1 mM ATP, 100 μ M GDP(β)S, 10 mM thymidine, 1 mM EDTA, 12.5 mM DTT, .025% SDS, 1.25 μ g activated PTX, 15 μ M [3 H] NAD (3 Ci/mmol), and 20-100 μ g of suspended or solubilized membrane protein. Reactions were carried out for one hour at 30°C and were terminated by heating at 100°C. G-proteins were separated by SDS-PAGE and were transferred to nitrocellulose and immunoblotted with an anti-Gi/O antibody. Immunoreactive bands of 40 Mr were cut out, dissolved in scintillation fluid, and were counted for 3 H. Incorporation of [3 H]-ADP-ribose into isolated Gi/O was 20-30 fold higher using solubilized versus suspended membranes. ADP-ribosylation of spinal cord membrane Gi/O was reduced by 62% in samples taken from mice 7 days after an intrathecal injection of 0.3 μ g/Kg PTX. ADP-ribosylation of cholate-solubilized membranes using [3 H] NAD coupled with scintillation counting of immunoblot bands provides a rapid, sensitive and quantitative method not requiring the use of 32 P.

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THE STRIATAL MUSCARINIC RECEPTOR INHIBITING DOPAMINE D₁-STIMULATED ADENYLYL CYCLASE ACTIVITY AS A TARGET FOR ANTICHOLINERGIC ANTIPARKINSON DRUGS.

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Activation of striatal muscarinic receptors inhibits the stimulation of adenylyl cyclase by dopamine (DA) acting on D₁ receptors. Recent studies have shown that full DA D₁ receptor agonists can improve the motor disturbances in animal models of Parkinson's disease. Thus, it is possible that the blockade of the muscarinic receptors inhibiting the striatal DA D₁ receptor activity may contribute to the antiparkinsonian action of antimuscarinic drugs. In this report, we show that various antimuscarinic drugs, currently used in the treatment of Parkinson's disease, potentially antagonize the carbachol (CCh) inhibition of rat striatal D₁-stimulated adenylyl cyclase activity. Drug Ki values (nM) are: benztropine 2.4, biperiden 3.1, trihexyphenidyl 5.0, procyclidine 8.2, ethopropazine 24.6, orphenadrine 41.5, diphenhydramine 164. There is a good correlation between the drug rank order of potencies and their clinical efficacies ($r = 0.970$; $P = 0.0003$). On the other hand, the drugs are weaker antagonists of the CCh stimulation of phosphoinositide hydrolysis in rat striatum, displaying the following Ki values (nM): benztropine 48.8, biperiden 9.6, trihexyphenidyl 31, procyclidine 98, ethopropazine 175, orphenadrine 608, diphenhydramine 2260. These potencies poorly correlate with the clinical efficacies of the drugs ($r = 0.776$; $P = 0.04$). The data support the possibility that the antagonism of the muscarinic inhibition of striatal DA D₁ receptor activity may be one of the mechanisms by which anticholinergic drugs exert their antiparkinsonian effect.

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TGF β AND FGF-2 DOWN-REGULATE m2 MUSCARINIC RECEPTOR mRNA EXPRESSION IN EMBRYONIC CHICK VENTRICULAR MYOCYTES.

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In previous studies we observed that exposing cultured embryonic chick cardiomyocytes to FGF-2 or TGF- β 1, but not IGF-I or PDGF, produced a dose-dependent decrease in the number of muscarinic receptors present on the cell surface. We developed an RNase protection assay using antisense riboprobes for chick cardiac cm2 and cm4 receptors (obtained from N. Nathanson, Seattle) to determine whether this decrease could be due to a growth factor induced decrease in the transcription rate for these subtypes. We found that both FGF-2 and TGF- β 1 decreased mRNA levels for cm2 receptors by 40-50% over the course of 48 hr exposure to either growth factor. This decrease is maintained out to 72 hr in cells exposed to either TGF- β 1 or FGF-2. These results closely match the magnitude and time course of our observations for growth factor induced decreases in cell surface [3 H]NMS binding. In contrast, while FGF-2 exposure for up to 72 hr produced no change in the amount of cm4 mRNA detected, TGF- β 1 exposures of 48 or 72 hr induced a 20-25% decrease in cm4 mRNA levels. These results suggest that cardiac muscarinic receptor expression in embryonic chick cardiomyocytes can be inhibited by specific peptide growth factors at the level of gene transcription.

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REGULATION OF THE CHICK M2 PROMOTER BY THE GATA FAMILY OF TRANSCRIPTION FACTORS AND BY CYTOKINES.

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We have isolated genomic regions containing the putative chick m2 (cm2) promoter and show regulation of cm2 promoter constructs by the cytokines leukemia inhibitory factor (LIF) and ciliary neurotrophic factor (CNTF). The cm2 promoter is also regulated by the GATA family of transcription factors.

Transfection of constructs containing the cm2 promoter driving the expression of the firefly luciferase gene into a human choriocarcinoma cell line (JEG-3) results in luciferase expression levels similar to those seen with vector alone.

Co-transfection of the cm2 firefly reporter constructs with an expression vector containing chick GATA-6 results in a dramatic increase in luciferase activity. Currently we are determining the specificity of the GATA factor(s) involved in this regulation. The proximal promoter region of the cm2 gene contains several potential GATA factor binding sites. We are investigating if chick GATA factors will bind these sites *in vitro*.

Using chimeric receptors containing the extracellular region of the GCSF receptor fused to the intracellular region of gp130, the common signaling subunit shared by the LIF and CNTF receptor, we are mapping cytoplasmic regions of gp130 involved with induction of the cm2 promoter by cytokines. We are also defining region(s) of the cm2 promoter necessary for cytokine regulation.

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38**RAPID DESENSITIZATION OF PHOSPHOINOSITIDE RESPONSES FOLLOWING ACTIVATION OF MUSCARINIC m3 RECEPTORS BUT NOT GnRH RECEPTORS IN α T3-1 CELLS IMPLICATES A RECEPTOR DEPENDENT MECHANISM.**

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There is now substantial evidence that many plasma-membrane heptahelical receptors linked to the activation of phosphoinositidase C, including muscarinic m3 receptors, undergo rapid desensitization within seconds of agonist exposure. This is reflected by a biphasic accumulation of [3 H]inositol phosphates in Li^+ -blocked cells and a transient peak of $\text{Ins}(1,4,5)\text{P}_3$ accumulation. A number of mechanisms have been implicated in this phenomenon although agonist-dependent receptor phosphorylation or depletion of the lipid substrate ($\text{PtdIns}(4,5)\text{P}_2$) appear the most likely. In contrast, gonadotropin-releasing hormone (GnRH) receptors in an immortalized mouse pituitary gonadotrope cell line, α T3-1, do not display features of rapid desensitization. We have used α T3-1 cells expressing recombinant human muscarinic m3 receptors to examine whether this is a receptor or cell dependent phenomenon and to gain further insight into the mechanism of desensitization. The results indicate that desensitization is receptor-dependent and suggest that availability of $\text{PtdIns}(4,5)\text{P}_2$ does not underlie the rapid partial desensitization of phosphoinositide responses following activation of muscarinic m3 receptors in these cells.

39**INSURMOUNTABILITY OF THE RECEPTOR-ANTAGONIST INTERACTION**

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Our previous results from the human neuroblastoma cells have suggested that the muscarinic antagonists atropine, NMS and 4-DAMP display insurmountable antagonism on carbachol induced Ca^{2+} mobilization, with an indication of a greater surmountability on Hm3 receptors. To study this further, we have expressed the muscarinic receptor subtypes Hm1, Hm3 and Hm5 in Sf9 insect cells. Stimulation with carbachol caused an increase in intracellular Ca^{2+} , which could be inhibited by the muscarinic receptor antagonists. Three different patterns of inhibition could be resolved for the different antagonists and receptor subtypes: i) surmountable inhibition with a parallel right-shift of the agonist dose-response curve, ii) insurmountable inhibition with a right-shift of the agonist dose-response curve and depression of the maximum signal, and iii) an intermediate pattern where the depression of the maximum signal reached a maximum at higher [antagonist]. The results indicate that atropine is more surmountable in Hm3 than in Hm1 or Hm5 receptors, and that NMS is more surmountable in Hm1 and Hm3 than in Hm5 receptors. 4-DAMP was completely surmountable in all the receptor subtypes studied. A direct correlation between the type of inhibition and the measured dissociation of the antagonists was observed. All the three different modes of inhibition could be reproduced in kinetic simulations by varying the dissociation rates of the antagonist-receptor complex. The simulation, however, required larger differences in dissociation rate constants of the antagonists than the measured ones. Thus, even though the differences in the dissociation rates could theoretically explain this phenomenon, the actual situation is more complex.

40**FUNCTIONAL PROPERTIES OF HUMAN MUSCARINIC RECEPTORS HM1, HM3 AND HM5 EXPRESSED IN INSECT CELLS**

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We have used the baculovirus expression vector system for functional expression of human mAChR subtypes m1, m3 and m5 in Sf9 insect cells. All three subtypes responded to carbachol with an increase in IP₃ and elevation of intracellular free Ca²⁺. The Ca²⁺ increase was fairly sustained and largely due to influx. Parallel measurements of [³H]NMS binding and Ca²⁺ increases at different infection times enabled us to compare the EC₅₀ for carbachol-mediated Ca²⁺ mobilization among subtypes and to see how the receptor levels influence agonist potency. The EC₅₀ values for Hm3 and Hm5 receptor subtypes were similar and were not significantly affected by change in receptor density. The EC₅₀ for Hm1 was somewhat higher than for the other two subtypes at low receptor density but decreased as the receptor density increased.

The receptor subtypes displayed no differences in their response to oxotremorine-M, which behaved as a full agonist. Oxotremorine and pilocarpine were weak partial agonists, but the maximal response to oxotremorine increased with the Hm3 subtype as the receptor level increased.

When tested in a whole cell cAMP assay the Hm1, 3 and 5 receptor subtypes did not affect the basal cAMP levels whereas forskolin- or cholera toxin-stimulated cAMP production was further stimulated through a mechanism involving Ca²⁺.

41**AGONIST-INDUCED ENDOCYTOSIS OF MUSCARINIC RECEPTORS: COUPLING EFFICIENCY TO PHOSPHOINOSITIDE HYDROLYSIS.**

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The functional status of internalized muscarinic cholinergic receptors (mAChRs) in SH-SY5Y neuroblastoma cells has been evaluated following the isolation of plasma membrane (P₁) and 'light' vesicular (V₁) fractions by means of differential centrifugation. The addition of oxotremorine-M (oxo-M) to SH-SY5Y cells resulted in a time-dependent (t_{1/2}=10 min) 4-fold increase in mAChR density in the V₁ fraction, with a corresponding loss of receptors from the P₁ fraction. mAChRs present in the V₁ fraction were readily recognized by lipophilic, but not hydrophilic, radiolabeled antagonists. When V₁ fractions from either control or oxo-M-pretreated cells were incubated with [γ -³²P]ATP, relatively little labeling of phosphatidylinositol 4,5-bisphosphate (PIP₂) was observed (<25% of that for P₁). In contrast, the labeling of phosphatidylinositol 4-phosphate (PIP) was comparable in the two fractions. Little PIP-kinase and phospholipase C (PLC) activity could be detected in the V₁ fractions, whereas PI-kinase activity in V₁ was 2-fold enriched over P₁. Immunoblot analyses indicated that, following oxo-M addition, G α_q /11 was increased by 53-61% in the V₁ fraction and that significant PLC β and PLC γ immunoreactivity was present in the same fraction. These data suggest that agonist addition to SH-SY5Y cells results in an endocytosis of both mAChRs and G α_q /11 into a vesicular fraction in which only a limited synthesis of PIP₂ can occur. Furthermore, although PLC β and γ isoforms can be identified in this fraction, they do not appear to possess significant catalytic activity. (Supported by NIH NS 23831 and NIMH MH 42652.)

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ACTIVATION OF MUSCARINIC RECEPTORS IN SH-SY5Y NEUROBLASTOMA CELLS ELICITS A WORTMANNIN-SENSITIVE TYROSINE PHOSPHORYLATION OF FOCAL ADHESION KINASE

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Human SH-SY5Y neuroblastoma cells have been utilized to investigate the mechanism whereby agonist occupancy of muscarinic receptors elicits an increase in the tyrosine phosphorylation of focal adhesion kinase (p125^{FAK}). SH-SY5Y cells express muscarinic cholinergic receptors (mAChRs) of predominantly the m₃ subtype, which are robustly coupled to phosphoinositide (PPI) hydrolysis and Ca²⁺ homeostasis. The addition of oxotremorine-M (oxo-M) resulted in an increased tyrosine phosphorylation of p125^{FAK} that was maximal by 10 minutes and persisted for 2 hours. Half-maximal phosphorylation of p125^{FAK} was observed at an oxo-M concentration of approximately 10 μ M. Oxo-M-stimulated p125^{FAK} phosphorylation was independent of the production of phospholipase C (PLC)-derived second messengers, integrin-extracellular matrix interactions, and cell attachment, but required an intact actin microfilament network. m₃AChR-mediated p125^{FAK} phosphorylation was attenuated by the addition of micromolar concentrations of wortmannin, a putative inhibitor of both phosphatidylinositol (PI) 3'-kinase and PI 4'-kinase. Wortmannin inhibited both receptor-stimulated p125^{FAK} phosphorylation and PPI hydrolysis with similar IC₅₀ values of approximately 1 μ M. These results suggest that although the formation of PLC-derived second messengers is not a prerequisite for p125^{FAK} phosphorylation following mAChR activation, inositol phospholipid turnover may nonetheless play a pivotal role in events that initiate the tyrosine phosphorylation of p125^{FAK}. (Supported by NIH NS 23831 and NIMH MH 46252.)

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AN M1 SELECTIVE AGONIST AF102B, AS A POTENTIAL DRUG IN TREATMENT OF ALZHEIMER'S DISEASE: BIOCHEMICAL AND PHARMACOLOGICAL PROPERTIES.

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Deficit in cholinergic transmission, abnormalities in Tau phosphorylation and appearance of β -amyloid (A β) deposits in the brain play a major role in the pathology of Alzheimer's disease (AD). The present study expands previous data showing the effects of AF102B, an M1 selective agonist on amyloid precursor protein (APPs) secretion, Tau-1 immunoreactivity and neurotrophic responses as studied in PC12 cells stably transfected with M1 muscarinic receptors (mAChR, PC12M1 cells) and in primary embryonal rat brain cell cultures. In both cell models, AF102B elevated APP secretion and increased Tau-1 immunoreactivity. Moreover, AF102B was found to stimulate APP secretion and to increase Tau-1 immunoreactivity. The increment in APP secretion and in the neurotrophic responses in PC12M1 cells was augmented by nerve growth factor (NGF) suggesting a cross talk between tyrosine kinases receptors and M1 muscarinic receptors. In attempts to identify intracellular transduction pathways involved in these activities, we examined the effects of several signal transduction inhibitors/activators. The protein kinase inhibitor K252a was found to inhibit NGF induced neurite outgrowth while enhancing the effect induced by AF102B either alone or in concert with NGF. Muscarinic-induced APP secretion was stimulated by K252a and partially inhibited by the protein kinase C (PKC) inhibitor GF109203X. The inhibitor of *ras* activation FTS partially inhibited muscarinic-induced APP secretion, while genistein a tyrosine phosphorylation inhibitor, abolished completely the stimulated secretion. These results suggest that PKC-dependent and *ras*-dependent pathways operate in parallel and converge at a tyrosine phosphorylation site within the cascade leading from muscarinic stimulation to APP secretion. Similar effects occurring in PC12M1 cells, in primary cell cultures and in brain slices propose a possible *in-vivo* synergism between M1 agonists such as AF102B and endogenous growth factors leading to neurotrophic and neuroprotecting activities as well as to non-amyloidogenic APPs secretion. M1 selective agonists may be used both in the treatment and in delaying the progression of AD.

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INHIBITION BY CALMODULIN OR S100 PROTEIN OF AGONIST-DEPENDENT PHOSPHORYLATION OF m2 MUSCARINIC RECEPTORS BY G PROTEIN-COUPLED RECEPTOR KINASE 2

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Agonist- or light-dependent phosphorylation of muscarinic acetylcholine receptor m2 subtypes (m2 receptors) or rhodopsin by G protein-coupled receptor kinase 2 (GRK2) was inhibited by calmodulin or S100 protein in a Ca^{2+} -dependent manner. The phosphorylation was inhibited in the absence or presence of G protein $\beta\gamma$ subunits, although the inhibition was suppressed in the presence of excess $\beta\gamma$ subunits. The dose-response curve for stimulation by $\beta\gamma$ subunits of phosphorylation of m2 receptors and rhodopsin was shifted to the higher concentration of $\beta\gamma$ subunits by addition of Ca^{2+} -calmodulin or Ca^{2+} -S100 protein. The phosphorylation by GRK2 of a glutathione S-transferase fusion protein containing a peptide corresponding to the central part of the third intracellular loop of m2 receptors (I3-GST), which includes the phosphorylation sites by GRK2, was not affected by Ca^{2+} -calmodulin or Ca^{2+} -S100 protein in the presence or absence of $\beta\gamma$ subunits, but the agonist-dependent stimulation of I3-GST phosphorylation by an I3-deleted m2 receptor mutant in the presence of $\beta\gamma$ subunits was suppressed by Ca^{2+} -calmodulin. These results indicate that Ca^{2+} -calmodulin or Ca^{2+} -S100 protein do not directly interact with the catalytic site of GRK2 but inhibit the kinase activity of GRK2 by interfering with the activation of GRK2 by agonist-bound m2 receptors and G protein $\beta\gamma$ subunits.

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EFFECTS OF G PROTEIN-COUPLED RECEPTOR KINASES ON DESENSITIZATION OF MUSCARINIC RECEPTORS

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Agonist-dependent sequestration of human muscarinic acetylcholine receptor (mAChR) m1-m5 subtypes was examined in COS-7 cells transiently expressing mAChRs with or without G protein-coupled receptor kinases (GRK2, 5 and 6) or GRK2 dominant-negative mutant (DN-GRK2). The amounts of sequestered mAChRs m2 and m4 subtypes were increased or decreased by coexpression of GRK2 or DN-GRK2, respectively. Effective concentrations of carbamylcholine also decreased by coexpression of GRK2. Sequestration of m1, m3, and m5 subtypes was hardly observed in control cells, but was markedly enhanced by coexpression of GRK2. Sequestration of all subtypes was not affected by coexpression of GRK5 and GRK6, except that sequestration of m2 subtypes expressed in BHK-21 cells was facilitated by coexpression of GRK5. Down regulation and uncoupling were examined on m2 subtypes stably expressed in CHO-K1 cells with or without GRK2. Down regulation as well as sequestration were found to be facilitated by coexpression of GRK2. Uncoupling of m2 subtypes from G proteins was assessed by measuring GTP γ S binding activity in membrane fractions after treatment of cells with carbamylcholine. The carbamylcholine-stimulated GTP γ S binding activity was markedly reduced by the pretreatment of cells with carbamylcholine, and the degree of reduction was further enhanced by coexpression of GRK2. This reduction of carbamylcholine-stimulated GTP γ S binding activity became not observed by treatment of cells with sucrose, which is known to suppress the sequestration. These results suggest that the apparent uncoupling is at least in part due to the sequestration of m2 receptors.

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OVEREXPRESSION OF β -ADRENERGIC RECEPTOR KINASE ATTENUATES MUSCARINIC m1 RECEPTOR COUPLING IN HEK 293 CELLS.

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β -Adrenergic receptor kinase (β ARK or GRK2) has been shown to desensitize a broad family of receptors, but its role in regulating human muscarinic hm1 receptor is not clear. To assess the possible role of GRK2 in agonist-promoted muscarinic receptor desensitization, we employed HEK 293 cells transfected with expression vector encoding the hm1 receptor and vectors encoding GRK2 or dominant-negative GRK2-K220W. A panel of mutants was used to study the possible phosphorylation sites on hm1 receptor and the effect of GRK2 on other regulatory events. Our results indicate that the coexpression of hm1 with GRK2 attenuates receptor-mediated PI turnover, whereas coexpression with GRK2-K220W was significantly less effective. These results suggest that intact catalytic activity of GRK2 is required for at least part of the observed reduction in coupling, measured by accumulation of IP. Several mutants studied failed to define potential sites for phosphorylation on hm1. (Supported by NIH grant GM43102 and MH00996)

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AGONIST-DEPENDENT PHOSPHORYLATION OF THE M2 MUSCARINIC ACETYLCHOLINE RECEPTOR: TWO PHOSPHORYLATION DOMAINS POSSESS DISTINCT REGULATORY ROLES.

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Acute exposure of the m2 subtype of muscarinic acetylcholine receptor (m2 mAChR) to agonist causes it to become rapidly desensitized in its ability to cause attenuation of adenylyl cyclase activity. Phosphorylation of the agonist-activated receptor plays a crucial role in this regulation of m2 mAChR signaling. We have previously shown that deletion of amino acids 282-323, located in the third intracellular loop of the m2 mAChR, abolished agonist-induced receptor phosphorylation and desensitization seen following pre-exposure to the mAChR agonist carbachol and significantly reduced receptor internalization. We have extended these observations by constructing several point mutations within this region. We have identified two clusters of Ser/Thr residues that were sites for agonist-dependent phosphorylation. Mutation of either cluster alone caused no change in the extent of agonist-dependent receptor phosphorylation, but when both clusters were mutated, agonist-dependent phosphorylation was nearly abolished, suggesting that there are "redundant" sites for m2 mAChR phosphorylation. Similarly, receptor internalization was unaffected by mutation of either cluster but completely inhibited when both domains were mutated. However, these two domains were found to be significantly different in the extent to which they participated in m2 mAChR regulation. Mutation of residues 307-311 prevented all desensitization of receptor signaling, while mutation of residues 286-290 produced a receptor which did desensitize following agonist exposure. These data suggest that while these domains may serve as redundant targets for agonist-dependent phosphorylation and regulation of internalization, receptor desensitization requires phosphorylation of cluster 307-311.

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CHARACTERISATION OF THE MUSCARINIC RECEPTOR KINASE MRK.

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Previous studies from our laboratory have demonstrated that agonist mediated phosphorylation of the PLC-coupled m3-muscarinic receptor is mediated by a serine kinase distinct from second messenger regulated protein kinases and from β -adrenergic receptor kinase (1,2). We have recently purified a protein kinase of 40kDa from the porcine brain that is able to phosphorylate the m3-muscarinic receptor in membranes preparation from CHO-m3 cells in an agonist dependent manner (3). This prompted us to call the kinase "muscarinic receptor kinase" (MRK). Here we present evidence that the identity of MRK is casein kinase 1 α (CK1 α). Amino acid sequence analysis of two proteolytic peptide fragments from purified MRK revealed two sequences WYGQEK and IEYVHTK which corresponded exactly with sequences within the catalytic region of CK1 α . Purified MRK from porcine brain was able to phosphorylate casein and Ex-m3. Furthermore, during the purification of MRK the activity associated with Ex-m3 phosphorylation exactly correlated with the casein kinase activity. The cDNA for bovine CK1 α was expressed in Sf9 cells. High speed cytosolic extracts from infected Sf9 cells showed increased ability to phosphorylate casein and the bacterial fusion protein Ex-m3. Furthermore, the activity associated with phosphorylation of Ex-m3 and casein co-purified in a manner consistent with the chromatographic properties of MRK. These results strongly indicate that the kinase previously identified as muscarinic receptor kinase is casein kinase 1 α .

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MUSCARINIC RECEPTORS ACTIVATING CATIONIC CHANNELS IN SMOOTH MUSCLE

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Cationic current (I_{cat}) evoked by activation of muscarinic receptors with carbachol (CCh) was studied in single guinea-pig ileal smooth muscle cells using patch-clamp recording techniques. Symmetrical Cs⁺-containing solutions (124 mM) were used to isolate I_{cat} from K⁺ currents; divalent cations were removed from the external solution and intracellular free Ca²⁺ was strongly buffered to 100 nM to prevent Ca²⁺-dependent modulation of I_{cat} . The muscarinic receptors are linked to the cationic channel by a pertussis toxin-sensitive G-protein; adding 1 mM GTP to the pipette solution strongly reduced desensitization in this system allowing us to obtain in control experiments several similar dose-response curves (maximal cationic conductance, G_{max} , plotted vs. log[CCh]). The ileum contains both M₂ and M₃ receptors. Using selective antagonists we characterized the muscarinic receptor which is linked to cationic channel opening. The cells responded to CCh by generating I_{cat} with the mean EC₅₀ of 8.3 μ M and G_{max} of 30 \pm 2 nS (n=66). 4-DAMP and *p*-F-HHSD which have higher affinity for M₃ receptors at low concentrations did not affect the sensitivity to CCh but reduced G_{max} ; at concentrations high enough to affect M₂ receptors G_{max} was strongly reduced and the dose-response curve was shifted to the right and became flatter suggesting noncompetitive antagonism at the M₂ receptor. On the contrary, methoctramine and himbacine which have higher affinity for M₂ receptors produced a parallel shift of the dose response curve without reduction in G_{max} . Values of pK_b (derived from conventional Schild plots which had slopes not significantly different from 1) were about 8.0 for both antagonists suggesting competitive antagonism at the M₂ receptor. We conclude that cationic channel opening which is responsible for membrane depolarization is produced by activating the M₂ receptor population.

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MUSCARINIC RECEPTOR INHIBITION OF ATP-SENSITIVE K⁺ CHANNELS IN SWINE TRACHEAL SMOOTH MUSCLE.

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In this study we used the patch clamp technique to characterize a cromakalim-induced current and its regulation by acetylcholine (ACh) in isolated tracheal smooth muscle (TSM) cells. Cells were voltage-clamped in the whole-cell configuration at -80 mV while bathed in a 60 mM K⁺ solution. The equilibrium potential for K⁺ was approximately -22 mV. Cromakalim (10 μ M), a synthetic K⁺ channel opener, activated an increase in inward current which reached a steady-state ranging in amplitude from 33-292 pA (mean \pm SE=97.4 \pm 13.4 pA). This current was inhibited by the sulfonylurea glibenclamide (10 μ M), a recognized ATP-sensitive K⁺ channel antagonist. The cromakalim-induced current was also partially inhibited by ACh in a concentration-dependent manner. Percent inhibition of the cromakalim-induced current by ACh ranged from 23.6 \pm 14.9 (10 nM) to 73.9 \pm 4.6 (1 μ M). The effect of ACh was mimicked by the phorbol ester, phorbol 12-myristate, 13 acetate (PMA, 100 nM) which inhibited the cromakalim-induced current by 65.7 \pm 9.3%. Inhibition by both ACh and PMA was reduced in the presence of the protein kinase inhibitor staurosporine. Our findings confirm the presence of ATP-sensitive K⁺ channels in TSM cells, and demonstrate that these channels are regulated by muscarinic receptors, most likely through a protein kinase C-dependent pathway. These results suggest that ATP-sensitive K⁺ channels may play a role in the regulation of membrane potential and contractility in these cells. (Supported by DA05094, HL55547 and the MS Lung Assoc.)

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CONCENTRATION-DEPENDENT DESENSITIZATION. MUSCARINIC RECEPTORS INVOLVED IN Cl⁻ SECRETION AND ITS DESENSITIZATION

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1 The mechanisms behind concentration-dependent desensitization (auto-inhibition) of muscarinic-induced tracheal Cl⁻ secretion was studied by nine agonists. Cl⁻ secretion in hen tracheal epithelium was assessed as the bumetanide-sensitive short circuit current (I_{sc}). The Cl⁻ secretion was activated by stimulating a muscarinic receptor, mAChR, with agonists having the rank order of potency: oxotremorine M > methacholine > ACh = oxotremorine > arecoline > pilocarpine > carbacholine > McN-A-343 >> SP-TZTP. Oxotremorine, arecoline, pilocarpine and McN-A-343 were partial agonists, whereas methacholine, carbacholine, and oxotremorine M were full agonists.

2 At high muscarinic agonist concentrations the induced Cl⁻ secretion was desensitized by an inhibitory receptor activated by the single agonist with a rank order of potency: oxotremorine M >> oxotremorine = methacholine > ACh > arecoline > carbacholine, at variance with the stimulatory sequence.

3 Cl⁻ secretion elicited by pilocarpine and McN-A-343 showed only slight signs of concentration-dependent desensitization.

4 SP-TZTP, a potent and highly selective muscarinic M₁ receptor agonist, failed in the concentration range 0.01nM-10 μ M 1) to induce Cl⁻ secretion, 2) to interfere with Cl⁻ secretion stimulated by other agonists, and 3) to interfere with agonist-induced concentration-dependent desensitization, thereby excluding the presence of M₁ receptors involved in stimulation/ reduction of transepithelial Cl⁻ secretion in hen trachea.

5 Possible presence of muscarinic spare receptors was excluded by use of 4-DAMP mustard.

6 We conclude that activation and concentration-dependent desensitization (auto-inhibition) of Cl⁻ secretion take place through separate signalling pathways in hen trachea.

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REGULATION OF CA²⁺ INFLUX BY MUSCARINIC ACETYLCHOLINE RECEPTORS (mAChR) IN PC12D CELLS

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PC12D is a subline of the rat pheochromocytoma-derived cell line PC12 that is characterized by accelerated neuronal differentiation following exposure to nerve growth factor. We have previously shown that PC12D cells express mRNA's encoding the m1 and m4 subtypes of mAChR, and that the m1 subtype alone mediates the rapid induction of the immediate-early gene zif268 following exposure to muscarinic agonists. This gene induction depends upon semi-independent intracellular signalling pathways that require protein kinase C and the influx of extracellular calcium, respectively; release of calcium from internal stores by itself is not a sufficient stimulus for induction of the zif268 gene. In the present study we have investigated the mechanisms by which mAChR regulate Ca²⁺ influx using PC12D cells loaded with the fluorescent dye Fura-2. Our data suggest the following conclusions: 1) mAChR-mediated Ca²⁺ influx is a consequence of the emptying of internal Ca²⁺ stores. We find no evidence for "receptor-dependent" Ca²⁺ influx distinct from influx stimulated by thapsigargin, which depletes intracellular Ca²⁺ stores by inhibiting the Ca²⁺ pump of the endoplasmic reticulum. 2) Influx of Ca²⁺ following exposure to mAChR agonists or thapsigargin is significantly reduced by depolarization of the cell membrane. This effect is at least in part due to a reduction in the electrochemical driving force for Ca²⁺ entry into the cells. 3) Activation of mAChR inhibits L-type voltage-dependent calcium channels. This inhibition is a consequence of m1 mAChR-mediated activation of protein kinase C and is also observed following exposure to phorbol esters.

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PRESYNAPTIC MUSCARINIC M2 RECEPTORS DEPRESS GLUTAMATERGIC EPSCs IN RAT HYPOGLOSSAL MOTONEURONS (HMs).

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Cholinergic modulation of synaptic inputs is likely to be significant in state-dependent regulation of motor responses. Whole-cell patch-clamp recordings were made from young adult rat HMs (n = 17), visually identified using Nomarski optics and infrared transillumination of transverse brainstem slices. Glutamatergic excitatory postsynaptic currents (EPSCs), evoked by electrical stimulation lateral to the hypoglossal motor nucleus, were reversibly reduced in amplitude by bath application of carbachol (10 μ M, 37 \pm 3%, mean % of control \pm SD, n = 22), muscarine (10 μ M, 50 \pm 3%, n = 12) or physostigmine (200 μ M, 54 \pm 7%, n = 3), without significant change in holding current, input resistance or EPSC rise time and decay constant. The mean EC₅₀ for carbachol or muscarine was 152 nM and 704 nM respectively (n >= 3 for each dose). EPSC reduction was completely blocked by atropine (1 μ M, n = 11), while antagonist inhibition curve analysis found a high mean apparent affinity constant (pK_a) of 8.07 for methoctramine, a M2 antagonist, but a low mean pK_a of < 7.0 for pirenzepine, a M1 antagonist, showing that depression of evoked EPSCs was mediated by muscarinic M2 receptors. With TTX present, the amplitude of postsynaptic currents evoked in HMs by focal pressure injection of L-glutamate (0.5-1 mM) was not reduced by carbachol or muscarine (n >= 5), while the frequency of spontaneous glutamatergic miniature EPSCs was significantly (P < 0.01) reduced without change in amplitude by carbachol or muscarine, indicating that excitatory glutamatergic inputs to rat HMs are modulated by muscarinic receptors at a presynaptic site. Sequential blockade of N- and then P-type calcium channels did not significantly alter the reduction of the remaining EPSC by carbachol, suggesting that the mechanism underlying EPSC depression most probably involves steps in synaptic release occurring after calcium entry into the presynaptic terminal. Forskolin and SQ22536, which respectively activate and inhibit adenylyl cyclase, or H7, a protein kinase A/C inhibitor, did not occlude muscarinic reduction of the EPSC. Membrane-permeant cyclic monophosphate analogs (8-bromo-cAMP or -cGMP), a guanylyl cyclase inhibitor (LY83583), or an inhibitor of calmodulin kinase-dependent activation of cAMP-specific phosphodiesterase (calmidazolium) also did not occlude muscarinic effects on the EPSC. These data suggest that modulation of cAMP levels does not mediate muscarinic effects on synaptic transmission. A marked loss of tongue tone is seen during rapid eye movement sleep, when central cholinergic neurons are active. We suggest that cholinergic depression of excitatory synaptic drive to HMs may partially underlie this loss.

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54**VOLTAGE-GATED CALCIUM CHANNELS POTENTIATE MUSCARINIC RECEPTOR-MEDIATED CALCIUM RESPONSES IN SH-SY5Y HUMAN NEUROBLASTOMA CELLS**

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Muscarinic receptor-mediated Ca^{2+} elevations were investigated using the fura-2 method and image analysis. Typical responses consisted of an initial fast and transient phase followed by a sustained phase. Removal of external Ca^{2+} did not affect the magnitude of the initial phase of the signal whereas the sustained phase was abolished. Hence they may mainly consist of a release from intracellular stores and an influx, respectively. Depolarization of the cells with 30 mM KCl in the presence of external Ca^{2+} caused a small elevation of $[\text{Ca}^{2+}]_i$. In these conditions a considerable potentiation of the fast phase of muscarinic Ca^{2+} response was seen. The potentiation was dependent on the extracellular Ca^{2+} as it was abolished in the presence of antagonists of voltage-gated Ca^{2+} channels or by removal of external Ca^{2+} prior to KCl. Removal of external Ca^{2+} after the KCl-induced increase in $[\text{Ca}^{2+}]_i$ partially inhibited the potentiation. If Ca^{2+} was readded together with the muscarinic stimulation a maximum potentiation was seen. The results suggest that the muscarinic response is potentiated by a Ca^{2+} -mediated priming of both a fast and transient receptor associated Ca^{2+} influx pathway and release from the intracellular stores.

55**LOSS OF NEURONAL M2 MUSCARINIC RECEPTORS WITH VIRAL INFECTION IN CULTURED AIRWAY PARASYMPATHETIC NERVES.**

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Inhibitory M2 muscarinic receptors (M2R) on the parasympathetic nerves, which normally limit release of acetylcholine, are dysfunctional during parainfluenza virus infections (Br J Pharmacol 102:267). We investigated whether viral infection of cultured airway parasympathetic neurons, decreases the function and expression of inhibitory M2R.

Cultured guinea pig airway parasympathetic neurons were infected with parainfluenza type 1 (Sendai virus). Infection was confirmed by haemadsorption assay. 24 hours after infection, cultures were electrically stimulated (5Hz, 0.2msec, 30V, 5sec) and released acetylcholine measured using chemiluminescence. Released ACh is expressed as a % of total ACh, (determined by adding 3MKCl). Stimulation of uninfected cultures released 12% total ACh, while the infected cultures released 40% total ACh. Atropine increased release in uninfected, but not in the infected cultures, demonstrating functional M2 receptors in the uninfected, but not in the infected cultures.

M2R gene expression was measured using competitive reverse transcription-polymerase chain reaction (rtPCR). A 43-base portion of the M2R PCR product was excised to create a template of lower molecular weight. This was transcribed in vitro to synthesize cRNA that was 43-bases smaller than the original M2R RNA. RNA was extracted from control and parainfluenza virus infected nerve cells. Five hundred nanograms of RNA were added to internal standard cRNA (0.01-10 pg/sample). rtPCR was carried out on these mixtures, and the PCR products quantified by electrophoresis and densitometry. Competition for primers between the internal standard and the neuronal RNA yields bands of equal density when the input amount of M2R mRNA is equal to the input amount of internal standard. Viral infection decreased nerve cell M2R mRNA levels by 84%.

Thus increased release of acetylcholine by viral infection is due to loss of inhibitory M2 muscarinic receptor expression and function in cultured airway parasympathetic neurons.

56**M1-SUBTYPE MUSCARINIC ACETYLCHOLINE RECEPTOR ANTAGONISTS
REDUCE MATRIX PRODUCTION BY SCLERAL CHONDROCYTES FROM CHICKS**

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Administration of the mAChR antagonist atropine has been shown to reduce the progression both of human myopia and of myopia induced by form deprivation (FD) in chicks. Because mAChRs are present on several ocular tissues (e.g., the retina in both species, and the ciliary muscles in humans) and on several non-excitabile tissues (e.g., corneal epithelial cells), there are several possible mechanisms whereby antagonists might influence eye growth. We asked, could mAChR antagonists act via a direct effect on the chondrocytes of the chick sclera?

The elongation of the eye that accompanies FD in chicks is thought to be due to an increase in the synthesis of matrix proteoglycans by scleral chondrocytes. We used dissociated chondrocytes from normal and FD eyes to test the ability of mAChR ligands to influence scleral proteoglycan synthesis.

We found that mAChR antagonists inhibited proteoglycan synthesis in a dose-dependent manner, with a rank order of potency of atropine > pirenzepine = 4-DAMP >> gallamine, suggesting that mediation of scleral growth is probably by the M1 receptor subtype.

We conclude 1) that muscarinic antagonist treatments *in vivo* may act directly on scleral chondrocytes to inhibit growth, and 2) that M1 mAChRs could play a role in growth regulation of the sclera.

57**THE EFFECTS OF AF-DX 116, A CARDIOSELECTIVE MUSCARINIC ANTAGONIST, ON
THE NEGATIVE INOTROPIC ACTION OF SOMAN, A CHOLINESTERASE INHIBITOR**

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In superfused left atria from male guinea pigs, rabbits and rats, myocardial contractility was measured in gassed (95% O₂ and 5% CO₂) Krebs-Henseleit solution at 32°C before and after addition of soman (methylpinacolyl phosphonofluoridate) or acetylcholine and/or AF-DX 116 ([11-[2-[(diethylamino)-methyl]-1-piper-pyridinyl]acetyl-5,11-dihydro-6H-pyrido[2,3-b][1,4] benzodiazepin-6-one), a selective muscarinic M₂ antagonist. Concentration-response curves for control acetylcholine (1x10⁻¹³ - 1x10⁻² M) and soman (1x10⁻⁸ - 1x10⁻² M) were examined. The acetylcholine concentration-response curves seen in the left atria differed among the guinea pig, rabbit and the rat, suggesting interspecies variation. Increasing AF-DX 116 to 1 x 10⁻⁴ M reversed the negative inotropy caused by soman in the rat heart. In a concentration-dependent manner, AF-DX 116 reversed the negative inotropy of acetylcholine and increased the inotropic response approximately 100% and 50% above the baseline contractile force in the guinea pig and rabbit, respectively. In addition, the responses in atria of three species rat, rabbit, and guinea pig were examined in the presence and absence of AF-DX 116 alone. Differences in responses were observed suggesting that the M₂ response pattern on the left atria is species-dependent. The actions of AF-DX 116 led to a reversal of the negative inotropic response in the rat atria, which suggests that M₂ receptor blockers may provide selective therapeutic relief for organophosphonate (i.e., soman) intoxication.

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STUDIES ON THE MUSCARINIC RECEPTOR SUBTYPE IN CHICK ILEUM.

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The muscarinic receptor initiating contraction in the chick ileum was previously suggested to be a functional correlate of the m5 gene product (Darroch & Mitchelson 1995). Functional studies using carbachol as agonist revealed antagonist affinity profiles with a high correlation to those reported for the cloned m5 receptor. Evaluation of the receptor subtype present in the ileum has been extended to binding and molecular analysis studies. Homogenates of whole ileum were prepared in 50mM Na phosphate buffer (pH 7.4) for binding studies at 37°C. Binding of [³H]-QNB revealed a K_D value of 0.158 ± 0.063 nM with a B_{max} of 419.4 ± 39.7 fmol/mg of protein. Pirenzepine and himbacine were found to displace [³H]-QNB binding from a single site with pK_i values of 6.85 and 7.24 respectively. *p*-Fluorohexahydrosiladifenidol also displaced from a single site with a pK_i value of 6.46. These profiles support functional studies that suggested the receptor has a different affinity profile to mammalian M₁, M₂, M₃ or M₄ receptors. Complementary molecular studies are being undertaken.

Darroch S and Mitchelson F, (1995) Life Sci, 56, abstract 78

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INVOLVEMENT OF MUSCARINIC ACETYLCHOLINE M₂ AND M₃ RECEPTORS IN SMOOTH MUSCLE OF THE TAENIA CAECI

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In the guinea-pig taenia caeci, as in the ileum, there is a preponderance of muscarinic acetylcholine M₂ receptors which are coupled to inhibition of adenyl cyclase, although M₃ receptor activation produces the contractile response. While M₃ receptors are coupled to the phosphatidylinositol (PI) pathway, there is also evidence for tyrosine kinase involvement. Genistein (20 µM), an inhibitor of tyrosine kinase, caused *ca* 30-fold rightwards shift of the concentration-response curve to acetylcholine. Histamine curves were also shifted by genistein *ca* 10-fold, but curves for K⁺ or Ba²⁺ were only shifted 2 to 3-fold, suggesting a selective action of genistein on acetylcholine and histamine. Studies were also conducted to evaluate the role of M₂ receptors in the taenia which differs from the ileum in possessing a high level of intrinsic tone. M₃ receptor inactivation by 4-DAMP mustard in the presence of methoctramine to protect M₂ receptors, was performed on the taenia. Treatment of the taenia with 4-DAMP mustard (60 nM) plus methoctramine (1 µM) for 1 hour led to a 20-fold shift in the concentration-response curve for acetylcholine. Following repeated washing, the tissue was either treated with isoprenaline or isoprenaline plus histamine. Methoctramine (0.1 µM) produced negligible shift of the concentration-response curve to acetylcholine in the presence of isoprenaline alone. However, methoctramine in the presence of histamine plus isoprenaline, shifted the curve for the neurotransmitter with an apparent pK_B of 7.3, suggesting histamine was integral to the involvement of M₂ receptors in the contractile response after M₃ receptor inactivation.

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NO INVOLVEMENT OF M₂ TYPE RECEPTORS IN DIRECT CONTRACTION OF GUINEA PIG TRACHEAL SMOOTH MUSCLE

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Cholinergic contraction of guinea pig tracheal smooth muscle preparations *in vitro* is mediated by M₃ type muscarinic receptors under normal conditions; an additional involvement of M₂ type receptors has been demonstrated after selective inactivation of M₃ receptors and under elevated intracellular cAMP levels and adjusted smooth muscle tone (Thomas & Ehlert, *Biochem. Pharmacol.* 1996;51:779). M₂ receptors have also been implied, however, in contraction under normal conditions (Kume et al, *Am.J.Physiol.* 1995;268:L221), in relatively young animals (250-350g) and using pertussis toxin or AF-DX 116. We therefore established AF-DX 116 and methoctramine affinity estimates (pK_B) using concentrations that do not significantly block M₃ receptors (0.1 and 0.3 μ M; 0.3 and 1.0 μ M, respectively), in addition to higher concentrations (1 and 3 μ M; 3 and 10 μ M). Contractions were measured in single ring preparations using isotonic recording, 60 min antagonist preincubation and methacholine as the agonist. It was found that, both in young (338 \pm 15g, n=3) and older (744 \pm 74g, n=3) animals, the lower concentrations of AF-DX 116 yielded significantly higher pK_B values than the higher concentrations, but that this was not the case with methoctramine (Table). The results with AF-DX 116 might indeed be taken to suggest a role for M₂ receptors in contraction under these conditions, although even pK_B values of 6.7 are on the low side. The results with methoctramine, which has higher M₂/M₃ selectivity, do not indicate the involvement of M₂ receptors at all. It is therefore concluded that the involvement of M₂ type receptors in guinea pig tracheal smooth muscle contraction *in vitro* under normal conditions is unlikely.

Apparent pK _B values	Young	Old
AF-DX 116 0.1-0.3 μ M	6.66 \pm 0.07 (4)*	6.64 \pm 0.09 (4)**
AF-DX 116 1.0-3.0 μ M	6.35 \pm 0.07 (6)	6.22 \pm 0.06 (6)
Methoctramine 0.3-1.0 μ M	5.55 \pm 0.03 (5)	5.73 \pm 0.08 (4)
Methoctramine 3.0-10 μ M	5.40 \pm 0.12 (5)	5.54 \pm 0.10 (5)

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FUNCTIONAL CHARACTERIZATION OF POSTJUNCTIONAL MUSCARINIC RECEPTORS IN RAT ANOCOCCYGEUS MUSCLE

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Muscarinic agonist	potency pD ₂	intrinsic activity
Methacholine	5.66	1.00
Carbachol	5.68	1.00
(+)-Muscarine	6.36	1.00
Oxotremorine M	6.21	1.00
APE	6.18	0.90
McN-A-343	3.89	0.68
4-Cl-McN-A-343	4.28	0.64

Postjunctional muscarinic receptors mediating contraction have been identified in the rat anococcygeus muscle (RAM) (Gillespie, *BJP* 45: 404, 1972). The aim of the present study was to characterize the muscarinic receptor subtype responsible for the contractile response in RAM by means of a battery of agonists and subtype-prefering antagonists.

RAM's were incubated at 37°C in Krebs buffer (1.8 mM Ca²⁺). Tetrodotoxin-insensitive (1 μ M) contractions to cumulative addition of the muscarinic agonists were measured isotonically. Antagonism (methacholine as agonist) was evaluated by Schild analysis.

Muscarinic antagonist	affinity pA ₂	slope
Pirenzepine	6.92	0.98
AQ-RA 741	6.75	1.06
p-F-HHSiD	7.68	1.04
Himbacine	7.11	1.00
R-(-)-HHD	8.52	0.98
S-(+)-HHD	6.06	0.99

The low potencies of the agonists, together with partial agonism observed, indicated a low effective receptor reserve associated with the contractile response. The Schild slopes for all antagonists investigated did not differ from unity, indicating their competitive nature. A comparison of the functional pA₂ values obtained in RAM with the binding affinities at native M1-M4 receptors (Pfaff et al., *Life Sci.* 56: 1038, 1995) clearly demonstrates that the muscarinic receptors mediating contraction in RAM are of the M₃ subtype (r = 0.95).

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MUSCARINIC M_2 RECEPTORS MODULATE RELAXANT RESPONSES TO ISOPROTERENOL IN RAT URINARY BLADDER

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We have shown previously that M_3 receptors mediate (+)-cis-dioxolane-induced contraction of the rat isolated urinary bladder (RIUB; (1)) despite the predominance of M_2 receptors in this tissue (2). As M_2 receptors are negatively coupled to adenylyl cyclase (AC), we sought to determine whether a functional role of M_2 receptors could be unmasked under conditions of elevated AC activity (isoproterenol (IPNE)-induced relaxation of KCl pre-contracted tissue).

(+)-Cis-dioxolane-produced concentration-dependent contraction of the RIUB ($pEC_{50}=6.09\pm0.23$). Under conditions of elevated AC activity (KCl (90mM) + IPNE (30 μ M)), the contractile potency of (+)-cis-dioxolane was unaffected ($pEC_{50}=6.19\pm0.21$). After preferential alkylation of M_3 receptors (exposure to 4-DAMP mustard (40nM) for 60 min in the presence of methoctramine (0.1 μ M) to protect M_2 receptors), (+)-cis-dioxolane produced concentration-dependent reversal (re-contraction) of IPNE-induced relaxation ($pEC_{50}=6.06\pm0.19$) but had little effect on pinacidil (1 mM) induced relaxation. The recontractions were antagonised by methoctramine and darifenacin, yielding pA_2 estimates of 6.79 ± 0.08 and 7.69 ± 0.13 , respectively, which were intermediate between those expected for these compounds at M_2 and M_3 receptors, consistent with the involvement of both these receptors.

These results suggest that, after preferential partial alkylation of M_3 receptors, one can demonstrate a functional role of M_2 receptors to reverse β -adrenoceptor mediated relaxation of the RIUB.

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PHARMACOLOGICAL, MOLECULAR, AND FUNCTIONAL CLASSIFICATION OF MUSCARINIC RECEPTOR SUBTYPES IN THE RAT URINARY BLADDER.

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To elucidate the function of muscarinic receptor subtypes in bladder contractility, we compared the effects of subtype selective muscarinic antagonists on carbachol induced and electric field stimulated contractility of rat bladder *in-vitro*. The affinity of a series of muscarinic antagonists derived from Schild plot analysis of cumulative carbachol dose response curves indicated M_3 mediated bladder contraction. Different affinities were observed with inhibition of electric field stimulated contractions. The M_2 selective antagonist methoctramine (10 nM) increased nerve evoked contractions by 17% ($p < 0.01$) whereas the M_1 selective antagonist pirenzepine (PZP, 10 nM) inhibited contractions by 18.5% ($p < 0.01$). Identical doses had no effect on carbachol induced contractions. Higher doses of all antagonists tested inhibited fully electric field stimulated contractions in a manner consistent with their affinity for M_3 receptors. These data indicate that presynaptic M_1 facilitory and M_2 inhibitory receptors and post-synaptic M_3 receptors interact to mediate bladder smooth muscle contraction. The presence of $m1$ receptors in the rat bladder body could not be demonstrated by subtype selective antibodies (only the $m2$ and $m3$ subtypes could be detected immunologically) and Northern blots did not reveal the presence of $m1$ receptor mRNA. However using the reverse transcriptase polymerase chain reaction (RT-PCR) $m1$, $m2$, $m3$, and $m4$ transcripts were identified in rat bladder. Predicted dose response curves based on a three receptor system were generated. Only the dose response curves generated by incorporating presynaptic M_1 facilitory and M_2 inhibitory receptors modulating an M_3 mediated contractile response resembled the electric field stimulated data. Addition of a presynaptic M_4 inhibitory receptor resulted in predicted dose response curves that were nearly indistinguishable from the curves predicted by the three receptor system.

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PRESYNAPTIC INHIBITORY MUSCARINIC AUTORECEPTORS MODULATING ^3H -ACETYLCHOLINE RELEASE IN HUMAN ISOLATED DETRUSOR MUSCLE

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The presence of presynaptic muscarinic mechanism(s) modulating the release of newly-synthesized tritiated acetylcholine (^3H -ACh) from cholinergic nerves was investigated in human isolated detrusor muscle strips. Specimens from the anterior part of the urinary bladder dome were obtained from patients undergoing total cystectomy due to bladder base malignancy. Muscular strips (20 mm long, 4 mm wide), prepared by removing serosal and mucosal layers and mounted isometrically in a modified Krebs solution (37°C), were preincubated with ^3H -choline and stimulated at 10 Hz to improve their neuronal ^3H -ACh content. Electrical field stimulation (EFS) induced contractile responses and tritium-outflow. These effects were prevented by TTX. Most of the evoked outflow was recognized as ^3H -ACh by HPLC analysis. When two EFSs (S_1 and S_2) were carried out at 5 Hz (600 pulses, 0.1 ms, 60 V) 30 min apart, an S_2/S_1 ratio of 0.84 was calculated. Muscarone (10-1000 nM), a potent muscarinic agonist, caused a concentration-dependent inhibition of the outflow of ^3H -ACh (pEC_{50} 7.30) with maximal inhibition by about 50%. Physostigmine (100 nM) markedly reduced (by 50%) the evoked release whereas atropine (100 nM) increased it by 25%. Maximal (50%) facilitation of release was observed for atropine when six intermittent EFSs were applied at 10 Hz (100 pulses, 1 ms, 12 V). Atropine and the subtype-selective antagonists 4-DAMP (M_3), methoctramine (M_2/M_4) and triptamine (M_2) increased the evoked release in a concentration-dependent fashion. The calculated pEC_{50} potencies were 9.07, 8.00, 7.97, and 8.73, respectively. These findings provide direct evidence that in the human urinary bladder acetylcholine release is under the local inhibitory control of muscarinic autoreceptors. The comparison between the observed rank order of pre- and postjunctional potency of muscarinic antagonists and their affinities at muscarinic receptor subtypes suggests that these autoreceptors may be regarded as M_2 or a mixture of M_2 and M_4 receptors.

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SPECIFIC AGONISTS FOR TWO SUBTYPES OF PRESYNAPTIC MUSCARINIC RECEPTORS WHICH REGULATE ACETYLCHOLINE RELEASE IN GUINEA PIG AUERBACH PLEXUS

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Several furan analogs of muscarine (M) were synthesized and evaluated pharmacologically to characterize the muscarinic receptors (MR) which regulate acetylcholine (ACh) release (1,2). Two furan analogs, 5-methoxyfurmethide (5-MOFT) and 5-hydroxymethylfurmethide (5-HMFT) indicated two subtypes of presynaptic MRs which regulate ACh release. 5-MOFT produced a contractile response of guinea pig longitudinal ileal muscle (EC_{50} 28mM), which disappeared upon depletion of tissue ACh with hemicholinium-3.

5-HMFT ($4 \times 10^{-8}\text{M}$) blocked nicotine-induced release of ACh and subsequent contraction of the muscle. The effects of both 5-MOFT and HMFT were blocked by atropine. These agonists did not exhibit significant effects on tissues containing M_2 or M_3 receptors. Both agonists were weak to induce phosphoinositol hydrolysis or attenuate cAMP accumulation in cultured human astrocytoma and neuroblastoma glioma cells, respectively (3,4). These results indicate that *Auerbach plexus* contains two novel muscarinic receptors (M_i , M_s), one excitatory and the other inhibitory, which maintain homeostasis of ACh release. (Supported by The Smokeless Tobacco Research Council, Inc.; The Council for Tobacco Research, U.S.A., Inc.; and The Study Center for Anesthesia Toxicology of Vanderbilt University.)

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CHARACTERIZATION OF PREJUNCTIONAL INHIBITORY MUSCARINIC AUTORECEPTORS IN GUINEA PIG TRACHEA USING TRIPITRAMINE

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Prejunctional inhibitory muscarinic autoreceptors on cholinergic nerve endings in guinea pig trachea have been widely taken as M_2 based on the increase in nerve or electrical field stimulation (EFS)-induced smooth muscle contraction with M_2/M_3 selective antagonists (cf. e.g. Ten Berge et al, Eur.J.Pharmacol. 1993,233:279). However, [3 H]acetylcholine release measurements under conditions of no auto-feedback, combined with Schild analysis, have indicated that M_4 receptors may be involved (Kilbinger et al, LifeSci. 1995,56:981). We therefore studied the effects of the new, M_2/M_4 selective muscarinic antagonist, tripitramine, on EFS-induced twitch contractions and on [3 H]acetylcholine release. Experiments were carried out as described in the respective above references; antagonist contact time per concentration was 15 min in contraction (tripitramine 0.1nM-3 μ M, cumulative administration) and 108 min in release experiments (tripitramine 30nM-1 μ M). It was found that tripitramine concentration-dependently increased twitch contractions from 1 nM onwards, with peak increase $202 \pm 15\%$ at 0.1 μ M and pEC_{20} (-log concentration yielding 20% of maximum increase; cf. Ten Berge et al, 1993) 8.29 ± 0.08 ($n=8$); similar pEC_{20} values were obtained with prolonged antagonist contact time (75 min per concentration). Release experiments yielded apparent pK_B 8.55 ± 0.05 ($n=19$, Schild slope constrained to unity), with slope 1.15 ± 0.11 (not different from unity). The results show that pK_B values correlate well with pEC_{20} values, but not with reported M_2 (approx. 9.5) or M_4 affinities (approx. 7.9). Correlation plots of apparent pK_B values at the prejunctional autoreceptor in guinea pig trachea and those at M_2 or M_4 receptors, using tripitramine, pirenzepine, himbacine, AQ-RA 741, hexahydrosiladifenidol and dicyclomine (cf. Kilbinger et al, 1995), yielded similar levels of agreement. This may indicate that pre-junctional muscarinic inhibitory autoreceptors in guinea pig trachea consist of a mixture of M_2 and M_4 receptors. Alternatively, since M_4 receptors and their mRNA have not been identified in guinea pig tracheal nerves after 1 week in culture, using receptor antibodies and PCR (Fryer & Jacoby, Am.J.Resp.Crit.CareMed. 1996,153: A844), the autoreceptors might be of the M_2 type, with atypical pharmacological properties.

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ANTISENSE OLIGODEOXYNUCLEOTIDE TO THE MUSCARINIC M_2 RECEPTOR SUPPORTS ITS ROLE AS A NEGATIVE AUTORECEPTOR REGULATING ACETYLCHOLINE RELEASE

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At least five (m_1 to m_5) distinct muscarinic receptors have been cloned thus far. However, it is difficult to ascertain their respective physiological roles due to the lack of highly selective agonists and antagonists for a given subtype. For example, the pharmacologically defined muscarinic M_2 class consists of two subtypes (m_2 and m_4 receptors), both having high affinity for the AF-DX series of antagonists. Recently, much interest has focused on the M_2 -like receptor family on the basis of its possible role as negative autoreceptor modulating acetylcholine (ACh) release in the brain. However, it is unclear if these autoreceptors belong to cloned m_2 or m_4 (or both) subtype(s). To address this issue, we utilized a combined antisense oligodeoxynucleotide-*in vivo* dialysis approach. Phosphorothioate-modified antisense oligodeoxynucleotides targeted to subtype specific sequences at the 5' region were continuously infused (1 μ g/ μ L/hr for 3 days) into the third ventricle of adult male Sprague-Dawley rats. These animals were then used in *in vivo* dialysis to monitor ACh release as recently described (Hersi et al., J. Neurosci. 15: 7150, 1995). The antisense against m_2 receptor subtype reduced AF-DX 384 (0.1 μ M)-induced hippocampal ACh release whereas the antisense against m_4 receptor subtype did not. It thus appears that the molecularly-defined m_2 receptors are particularly involved in the regulation of ACh release in the rat hippocampus. This work was supported by the MRCC.

68**MUSCARINIC RECEPTORS COLOCALIZE WITH PROTEINS IN CLATHRIN-COATED VESICLES.**

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To study muscarinic receptor internalization, we have stably transfected human embryonic kidney (HEK 293) cells with human muscarinic cholinergic receptor subtype 1 (hm1) epitope-tagged at the amino terminus. Using antibodies to this epitope, we have been able to visualize the location of the receptor in the cells. In the absence of agonist treatment, the receptors reside predominantly at the cell surface. Within a few minutes after addition of carbachol or other muscarinic agonists, the receptors redistribute into intracellular vesicles. We have performed double-labeling studies using antibodies against the epitope-tagged receptor together with antibodies to clathrin, α -adaptin, and the transferrin receptor in order to characterize the vesicles containing the internalized Hm1 receptors. We have demonstrated a strong colocalization following carbachol treatment between hm1 receptors and clathrin in intracellular vesicles suggesting that the pathway of agonist-induced internalization is clathrin-mediated. We have also shown colocalization in intracellular vesicles with α -adaptin, a subunit of the AP2 complex of clathrin-coated pits and vesicles, and with the transferrin receptor, supporting the role of clathrin in hm1 internalization. Presently we are looking at G proteins in order to determine if an association between the hm1 receptor and G proteins exists within intracellular vesicles. There is no apparent colocalization between the hm1 receptor and the α subunits of Gq/11, Gs, or Gi-1 in intracellular vesicles following short treatments with carbachol. (Supported by MH 00996, LMT supported by AFPE and GM08338.)

69**DIVERSITY OF mAChR HETERORECEPTOR PROTEINS IN MULTIPLE HIPPOCAMPAL CIRCUITS**

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Presynaptic mAChR heteroreceptors modulate the release of many neurotransmitters throughout the brain and the peripheral nervous system. Pharmacologically, these presynaptic receptors frequently have been identified as M₂-like, but little or no direct anatomical evidence exists identifying individual gene products on nerve terminals of specific neural circuits. Recent immuno-localization studies have revealed highly laminated patterns of m1-m4 protein expression in the hippocampus, suggesting possible presynaptic localizations. To test the hypothesis that some mAChR subtypes expressed in the molecular layer of the dentate gyrus are localized presynaptically on different afferent pathways that terminate there, we performed a series of physical and chemical lesions to cause specific degeneration of hippocampal commissural, associational and perforant pathways in rat brain.

In all lesions, m1 expression in the molecular layer was unchanged, indicating that m1 is not presynaptic expression in these pathways. An m2 immunoreactive band in the molecular layer was eliminated only by lesions of the associational pathway, consistent with m2 expression in hilar interneurons. m3 immunoreactivity in the molecular layer was decreased by lesions of the entorhinal cortex, indicating this subtype is presynaptic on terminals of perforant pathway projections. m3 was also decreased by lesions of both the commissural and associational pathways simultaneously suggesting it is also presynaptic on these afferents. Likewise, m4 immunoreactivity in the molecular layer was dramatically decreased by lesions of the commissural/associational and perforant pathways, indicating its presynaptic localization on terminals of all of these pathways. Therefore, this study provides evidence for the presynaptic localization of multiple mAChR subtypes (m2, m3 and m4) in the same pathway as well as the expression of mAChR heteroreceptors in multiple hippocampal afferents. Supported by RO1 NS30454 (A.I.L.) and NRSA MH1186-02 (S.T.R.).

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CHARACTERIZATION AND LOCALIZATION OF MUSCARINIC CHOLINERGIC RECEPTOR SUBTYPES IN THE HUMAN PLACENTA

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A placental cholinergic system has been demonstrated in different mammals including humans. However, no information is available concerning cholinergic receptors expressed by placenta.

In this study the subtypes and the localization of muscarinic cholinergic receptors expressed by frozen sections of human placenta were investigated. Radioligand binding assay and light microscope autoradiography techniques were used with [^3H]-N-methyl-scopolamine (NMS) as a radioligand. Characterization of M_1 - M_4 muscarinic cholinergic receptor subtypes was accomplished by incubating sections of placenta with [^3H]-NMS in the presence of appropriate concentrations of non-labelled compounds active on subtypes of muscarinic cholinergic receptors.

[^3H]-NMS was specifically bound to sections of human placenta. Analysis of [^3H]-NMS displacement curves revealed that placental tissue expresses the M_1 - M_4 subtypes of muscarinic cholinergic receptors. These sites were located primarily within syncytial cells of placental villi, but not in placental vasculature.

These findings indicate that human placenta is a source of the 4 subtypes of muscarinic cholinergic receptors demonstrable with radioligand binding techniques. Further work is in progress to evaluate the functional significance of these sites.

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DEMONSTRATION OF MUSCARINIC RECEPTOR mRNAs IN PARIETAL CELLS, ENTERIC GANGLIA AND IMMUNE CELLS OF RAT STOMACH BY IN SITU HYBRIDIZATION HISTOCHEMISTRY

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Five subtypes (M_1 - M_5) of muscarinic acetylcholine receptor (mAChR) mRNAs are mapped in the stomach of intact and immobilized rats by *in situ* hybridization histochemistry (ISHH). Frozen sections were hybridized with [^{35}S]-UTP labeled ribonucleotides directed against fragments of the M_1 - M_5 mRNAs. We failed to detect any mRNA encoding the M_2 , M_4 or M_5 mAChRs. M_1 mRNA was found over the intramural ganglionic cells, in some deep epithelial cells of the prepyloric area (most likely gastrin producing G cells), and in various cell types of the tunica propria (TP). No labeling was seen over the epithelial cells of the fundic mucosa. Immobilization (three hours on two consecutive days), which provoked deep erosions in the stomach, did not alter the distribution of M_1 mRNA. M_3 mRNA was detected in some parietal cells (immunostained by a monoclonal antibody), in chief cells, and in smooth muscle cells of the tunica muscularis. A large number of various cell types in the TP, and some intramural ganglia were densely labeled for M_3 mRNA, while only weak labeling was seen over the surface mucous cell layer. Immobilization increased the density of labeling in both epithelial and TP cells. These data confirm former observations, obtained by pharmacological and radio ligand binding assays, that M_3 is the major mAChR subtype in the stomach epithelium. Expression of M_1 and M_3 mRNAs in TP cells support the existence of a non-epithelial, non-neuronal cell regulatory system in the gastrointestinal tract.

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IN VITRO AUTORADIOGRAPHIC AND RADIOLIGAND BINDING ASSAY EVIDENCE FOR THE PRESENCE OF M2, M3, AND M4 mAChR SUBTYPES IN RAT SPINAL CORD

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Activation of muscarinic, cholinergic receptors (mAChRs) in spinal cord produces antinociception (*Anesthesiol.* 80:1338, 1994) and increased blood pressure (*Brain Res. Bull.* 27:47, 1991). These functional data encouraged the present study designed to identify and localize the mAChR subtypes in spinal cord. *In vitro* autoradiography performed on 5 thoracic spinal cords showed the presence of M2 receptors in Rexed laminae I-III (20-30 fmol/mg), IV, VI, VII, IX, and area X, and M3 receptors in laminae I-III, with the highest densities in laminae I/II (20-25 fmol/mg). M1 receptors were not detected. Saturation binding assays using [³H]-pirenzepine (PZ) in spinal cord homogenates confirmed the absence of M1 mAChRs. Competition binding using [³H]-N-methyl scopolamine ([³H]-NMS) and PZ yielded a competition curve with a Hill coefficient (n_H) of 0.95 ± 0.01 and a $K_i = 164.5 \pm 30.5$ nM, indicating the presence of M3 mAChRs. Competition of [³H]-NMS with methoc-tramine (METH) resulted in a competition curve ($n_H = 0.99 \pm 0.02$) with a $K_i = 24.5 \pm 1.5$ nM, indicating the presence of M2 mAChRs. AF-DX 116 and METH + atropine showed shallow competition curves with $n_H = 0.76 \pm 0.05$ and $n_H = 0.61 \pm 0.08$, respectively. Curve fitting analysis using the LIGAND program (*Analytical Biochemistry* 107:220-239) indicated that AF-DX 116 recognized M2 and M4 mAChRs, and that METH + atropine recognized M3 and M4 receptors. The finding that M2 and M3 receptors are localized to laminae I-III where nociceptive A δ and C fibers terminate is consistent with the possibility that these mAChR subtypes may modulate antinociception. Support: USPHS grant MH 45361 (HAB), SMRC B96-99Z-11159-02 (UH).

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CHARACTERIZATION AND IMMUNOCYTOCHEMICAL LOCALIZATION OF MUSCARINIC ACETYLCHOLINE RECEPTORS IN CHICK DORSAL ROOT GANGLIA.

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We have demonstrated that chick dorsal root ganglia (DRG) express several cholinergic markers such as ChAT, AChE, and the high affinity choline uptake system. The structural and functional heterogeneity of mAChRs and their ability to mediate several different responses in the SNC, SNP and in some peripheral organs suggested to investigate also the presence of mAChRs in DRG and their functional characterization. Binding studies performed on E18 DRG sections using a muscarinic antagonist [³H-QNB] have demonstrated the presence of mAChRs. Scatchard plot of saturation isotherms have revealed a dissociation constant (K_d) value of 0.75 ± 0.02 nM and a maximum density of binding sites of 7.2 ± 0.5 fmol/mg tissue. The use of selective ligands such as pirenzepine, methoctramine, carbachol, 4-DAMP and tropicamide has allowed to demonstrate the presence of M_1 , M_2 and probably M_4 receptor subtypes. Analysis of the second messenger pathways activated by selective muscarinic agonists has confirmed the pharmacological data. In fact, the carbachol (M_2 agonist) induced a decrease of cAMP levels both in basal conditions and after PGE₁ treatment. Oxotremorine (M_1 agonist) and muscarine, instead, caused a fast increase of IP₃ levels with consequent elevation of intracellular calcium. We have also performed mAChR immunolocalization on DRG sections dissected from E12, E18 and five days post hatching, both at light and electron microscope. The monoclonal antibody used was not selective for receptor subtypes. Light microscopy analysis has showed the presence of numerous immunopositive neurons, apparently differing in their size, location in the ganglion and staining intensity. Electron microscopy has revealed immunostaining mostly associated to Nissl bodies and Golgi apparatus. Myelinated and unmyelinated fibres, both in the central and peripheral projections, were also immunopositive, indicating a possible transport of mAChRs towards the spinal cord and the periphery, respectively. Perineuronal satellite cells and Schwann cells were intensely labeled, particularly at E12, showing a cytoplasmic staining sometimes associated with restricted areas of the plasma membrane.

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IN VIVO GLUCOCORTICOIDS DECREASE EXPRESSION OF MUSCARINIC RECEPTORS IN AIRWAY SMOOTH MUSCLE

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Airway smooth muscle (ASM) tone is the net result of forces promoting constriction and relaxation. Relaxation is promoted by activation of β_2 -adrenergic receptors which activate adenylyl cyclase, whereas the major constrictor pathway involves acetylcholine, which binds to both M_2 and M_3 muscarinic receptors. We questioned whether acute corticosteroid administration could have beneficial effects on muscarinic receptor pathways in ASM, and if so whether M_2 or M_3 muscarinic receptor subtypes decreased, and whether the effect was due to the glucocorticoid itself or a non-specific effect of the corticosteroid. 18 Basenji-greyhound dogs were randomly assigned to 4 groups for 3 days of daily subcutaneous injections; methylprednisolone (MPN) (2 mg/kg/day) (n=6), deoxycorticosterone (DOC) (0.5 mg/day) (n=5), the vehicle DMSO (0.2 ml/day) (n=3) or no treatment (n=4). Trachealis muscle homogenates were prepared and frozen (-70°C). Total muscarinic receptor numbers were determined by incubation with a saturating concentration of the non-selective muscarinic antagonist [^3H]-QNB (1 nM), \pm 2 μM atropine. The percent and antagonist affinities of M_2 and M_3 receptors was determined by competitive binding of tripitramine (10 μM - 0.1 nM) with 0.3 nM [^3H]-QNB. While DMSO and the mineralocorticoid DOC were without effects on total numbers of muscarinic receptors as compared to untreated animals ($p = 0.78$ and 0.53 , respectively) ($B_{\text{max}} = 578 \pm 53$, 565 ± 141 and 612 ± 20 fmol/mg protein for DMSO, DOC and untreated, respectively) MPN significantly decreased muscarinic receptor numbers (320 ± 17 fmol/mg protein) as compared to untreated animals ($p < 0.0001$). The percentage of M_2 and M_3 receptors and the affinity of each subtype for tripitramine were unaffected by MPN treatment, suggesting that glucocorticoid therapy reduced the number but not affinities of both M_2 and M_3 receptors. These results suggest that part of the beneficial effects of acute glucocorticoid administration in asthmatics may be a reduction in the numbers of M_2 and M_3 muscarinic receptors in airway smooth muscle, resulting in less opposition to β -adrenergic-mediated relaxation. Supported by NIH HL 45974.

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DIABETES PREVENTS LOSS OF NEURONAL M_2 RECEPTOR FUNCTION AND AIRWAY EOSINOPHILIA FOLLOWING ANTIGEN CHALLENGE IN THE RAT LUNG.

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In the lung, neuronal M_2 muscarinic receptors (nM2Rs) tightly control release of acetylcholine (ACh) from parasympathetic nerves. Loss of nM2R function has been observed in animal models of asthma as well as in human asthmatics, causing increased ACh release and increased broncho-constriction. This loss of function has been associated with airway eosinophilia, as eosinophil major basic protein is an allosteric antagonist at the nM2R. Asthma and diabetes, though both common diseases, do not frequently occur in the same patient (Abrahamson, 1941). This work investigates the effect of diabetes on nM2R function and airway inflammation following antigen challenge.

Rats were sensitized to 10mg/kg ovalbumin (OA) + 100mg/kg $\text{Al}(\text{OH})_3$ sc. On day 14, some of the rats were made diabetic via streptozotocin (65mg/kg iv) and some of the diabetic rats were treated with low doses of NPH insulin (2iu/day sc for 7 days). On day 21, the rats were challenged with aerosolized 5% OA for 5 min. Unchallenged, non-diabetic rats served as controls. Blood glucose levels were elevated in both diabetic (476 ± 25.1 mg/dL) and insulin-treated diabetic rats (419 ± 41.2 mg/dL) vs controls (162 ± 19.8 mg/dL). 18 hours later the rats were anesthetized, tracheostomized, ventilated and paralyzed. Vagus nerves were cut and stimulated to induce bronchoconstriction (VIBr, 30Hz, 25-40V, 0.4msec, 180 pulses/train).

In control rats, the nM2R agonist pilocarpine (0.001-100 $\mu\text{g/kg}$, iv) inhibited VIBr in a dose related manner. After antigen challenge (AgC), the nM2R was shown to be dysfunctional, as pilocarpine had no effect on VIBr ($p=0.0001$ vs controls). In contrast, nM2R function was protected from AgC in diabetic rats, but this protection was lost by the administration of insulin. Loss of nM2R function in AgC rats was associated with significant eosinophilia in bronchoalveolar lavage fluid (BAL, $p=0.003$ vs controls). However, little or no eosinophils were noted in BAL following AgC of diabetic rats. Eosinophil numbers in the BAL were restored by administration of insulin to AgC diabetic rats. Since this dose of insulin was not able to restore blood glucose to control levels, these changes can not be ascribed solely to an effect of hyperglycemia. These data imply a role for insulin in modulation of nM2R function and/or inflammation.

Abrahamson, E.M. (1941) *J. Clin. Endocrinol.* 1:402

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M₁-CHOLINOCEPTORS MEDIATE THE PRESSOR RESPONSES TO ELECTRICAL AND CHEMICAL STIMULATION OF AMYGDALA IN CONSCIOUS RATS

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The effects of the electrical and chemical stimulation of the central nucleus of the amygdaloid complex (CNA) on mean arterial blood pressure (MAP) were investigated in conscious, unrestrained Sprague Dawley rats. The electrical stimulation of the CNA led to reproducible increases (29.1 ± 5.6 mm Hg) in MAP. Atropine, pirenzepine or AF-DX 116 given into CNA blocked these blood pressure changes dose-dependently. Atropine and pirenzepine were found to be equipotent in this regard ($ID_{50}=1.05$ and 0.23 nmol, respectively) whereas AF-DX 116 was 140.3 times less potent than pirenzepine ($ID_{50}=39.5$ nmol). The chemical stimulation of CNA by 5 nmol carbachol caused 21.0 ± 3.5 mm Hg increase in MAP. Pirenzepine (icv) inhibited carbachol-induced pressor responses dose-dependently ($ID_{50}=7.99$ nmol) whereas AF DX 116 did not significantly alter those responses at 100 nmol. The high potency of pirenzepine compared with AF DX 116 indicates the involvement of M₁ muscarinic receptors located at CNA in the pressor responses induced by both the electrical and the chemical stimulation of this area.

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THE EFFECTS OF MUSCARINIC CHOLINERGIC ANTAGONISTS AND AGONISTS ON PREPULSE INHIBITION OF THE ACOUSTIC STARTLE REFLEX.

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Prepulse Inhibition (PPI), the reduction in startle response produced by presentation of a substartling prepulse stimulus, is disrupted in schizophrenics. Lesioning of cholinergic neurons in the brainstem reticular formation has also resulted in disruption of PPI (Koch et al., 1993), suggesting a possible cholinergic involvement in both normal sensorimotor gating and the pathophysiology of schizophrenia. In the present study, male Sprague-Dawley rats were administered systemic or intracerebroventricular injections of muscarinic antagonists and agonists to determine if dose-dependent effects on startle amplitude and PPI would be observed. Test sessions consisted of counterbalanced presentations of the following four trial types: no stimulus, startle pulse alone (106 db), prepulse alone (77 db), and prepulse+pulse. Nonselective muscarinic antagonists, such as scopolamine, benztropine, and trihexyphenidyl produced a significant dose-dependent disruption of PPI with no significant effect on startle amplitude. The muscarinic agonists oxotremorine, pilocarpine, RS-86, and arecoline had no effect on PPI. However, each agonist caused a significant decrease in startle amplitude at the highest dose tested. In further experiments, the interstimulus interval (ISI) between the prepulse and startle stimuli was varied from 30 to 1000 msec. The nonselective, muscarinic antagonists disrupt PPI in an ISI-dependent manner. The present findings suggest that the muscarinic cholinergic system may be involved in the characteristic cognitive flooding and fragmentation observed in schizophrenia.

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MUSCARINIC AGONISTS PRODUCE ANALGESIA THROUGH PERTUSSIS TOXIN-SENSITIVE MUSCARINIC RECEPTORS IN THE SPINAL CORD.

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Muscarinic agonists have been shown to produce antinociception in rodents when administered SC, ICV or IT. Two of the cloned muscarinic receptors (m2,m4) have been shown to be linked to inhibitory G-proteins, which when stimulated, lead to attenuation of cAMP production. In current studies, pertussis toxin (PTX) was employed to catalyze the ADP-ribosylation of G_i-proteins to determine if PTX sensitive muscarinic receptors are involved in antinociception. PTX was administered IT 7 days prior to systemic administration of muscarinic agonists, an alpha-2 adrenergic agonist or morphine. Analgesia testing was performed by measuring tail-flick latency using a 55 °C water bath whereas a 45 °C water bath was used to test for reversal of hyperalgesia induced by IT administration of PTX. Muscarinic agonists were more potent than morphine in producing analgesia when using the 55 °C water tail-flick test as well as reversing the hyperalgesia induced by intrathecal PTX. Clonidine was also more potent than morphine in producing analgesia and reversal of hyperalgesia. Muscarinic agonists and clonidine, when administered SC 30 minutes prior to measuring tail-flick latency using a 45 °C water bath, were fully efficacious in reversing PTX induced hyperalgesia, although 3 to 10-fold higher doses were required than for producing analgesia. Further, concomitant administration of muscarinic agonists by ICV and IT routes revealed synergistic interactions, as determined by isobolographic analysis, using a 55 °C water bath tail-flick test. Similarly, concomitant ICV and IT administration of morphine was synergistic in producing antinociception in the 55 °C tail-flick test. Taken together, these data suggest that the muscarinic receptors involved in producing analgesia in the spinal cord are linked to PTX-sensitive G_i-proteins and that they play a critical role in antinociception. Further studies using selective muscarinic antagonists should reveal which receptor subtype is involved in antinociception.

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MUSCARINIC ANTINOCICEPTIVE AGENTS WITH POTENT AND SELECTIVE EFFECTS ON THE GI TRACT: POTENTIAL APPLICATION FOR THE TREATMENT OF IRRITABLE BOWEL SYNDROME.

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Non-selective muscarinic antagonists are currently used as antispasmodic agents for the treatment of irritable bowel syndrome (IBS). Their efficacy is limited by typical anticholinergic side-effects. The non-selective antagonists provide little relief for the abdominal pain characteristic of IBS. In the present work, our goal was to find compounds with muscarinic activity that would have both antispasmodic and antinociceptive activity. The ferret was used for evaluating GI effects based on this species having intestinal hypermotility which closely resembles that seen in the human IBS patient. Compounds were evaluated for their ability to normalize hypermotility without increasing transmural potential difference. Antinociceptive activity was evaluated in the mouse writhing assay. LY316108 / NNC11-2192 was found to have the desired combination of antinociceptive and selective GI normalizing effects: mouse writhing ED₅₀ = 0.1 mg/kg (s.c.), inhibition GI motility ED₅₀ = 0.2 mg/kg (i.v.), potential difference ED_{1mV} > 30 mg/kg (i.v.). This desirable efficacy and selectivity profile indicates its potential for clinical evaluation in the treatment of IBS.

80**ANTICONVULSANT EFFECTS OF MUSCARINICS.**

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Compounds that increase muscarinic cholinergic tone such as arecoline and physostigmine may prolong onset and lower intensity of convulsions, and reduce mortality in mice given convulsive doses of pentylenetetrazole (PTZ; Ruossinov and Giorgiev, 1972). Seizures induced by the L-glutamate-1-decarboxylase (GAD) inhibitor isoniazid were blocked by muscarinic agonists (Sauerberg et al., 1986). To explore the anticonvulsant potential of muscarinic agonists, we compared three 1,2,5-thiadiazol analogs with pilocarpine, arecoline, oxotremorine and RS86 against convulsions induced by isoniazid, PTZ, the inverse benzodiazepine agonist DMCM, the Excitatory Amino Acid (EAA) agonists NMDA, kainic acid, and AMPA, Maximal Electro Shock (MES), and audiogenic seizures in DBA/2 mice. Motor performance was measured on a rotarod. The muscarinic standards and the 1,2,5-thiadiazole analogs were all effective against isoniazid and MES induced seizures. The 1,2,5-thiadiazoles were partially effective against PTZ but inactive against EAA agonist induced seizures. In line with findings that muscarinic agonists may decrease glutamate release in the hippocampus (Marchi and Raiteri, 1989), one 1,2,5-thiadiazol inhibited glutamate mediated excitatory postsynaptic potentials in the CA1 region of the hippocampus. We hypothesize that the decreased levels of GABA caused by isoniazid shifted the balance in favor of the EAA's resulting in convulsions. By decreasing glutamate release, the muscarinic agonists acted to restore the GABA / EAA balance and thereby attenuated the convulsions.

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81**M₂ BINDING AND m2 RECEPTORS IN EXPERIMENTAL MONKEY NEOCORTEX**

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M₂ binding modulates presynaptic ACh release but is not altered in rodents with lesions that remove all cholinergic inputs to posterior cingulate cortex (PCC), including layer V where these axons terminate. Since M₂ binding and m2 receptors may be postsynaptic in primates, changes in M₂ binding in Alzheimer's disease (AD) may not be due to presynaptic, cholinergic changes. Unilateral cingulotomy lesions were placed in 5 monkeys and PCC assayed with AF-DX 384 binding in competition with pirenzepine (PZ). Under autoradiographic conditions (i.e., 2 nM AF-DX 384; 100 nM PZ) PZ-insensitive binding is comprised of 65% m2 and 24% m4 binding based on published K_d values and m1-m5 receptor densities for human cortex. Immunohistochemistry for choline acetyltransferase (ChAT) showed that there are no intrinsic cholinergic neurons in PCC and that the lesion completely removed ChAT-ir axons. In 3 animals, the K_H was 4.97±1.5 nM and the K_L was 210±43. The density of PZ-insensitive AF-DX 384 binding was not changed. The B_{max} in control hemispheres was 164±31 fmol/mg protein and in ablated hemispheres it was 186±26. Autoradiography of PCC allows for laminar analysis of binding in control and ablated hemispheres. No changes in binding were detected in any layers, including layer V. Finally, reverse transcriptase-PCR was used to isolate m2 mRNA from control and ablated hemispheres. There were no changes in mRNA levels for this receptor in the ablated hemisphere. **CONCLUSIONS:** 1) Presynaptic M₂ binding and m2 receptors do not make a major contribution to total M₂ binding in primate PCC. 2) Most changes in M₂ binding in AD (increases and decreases) may be due to postsynaptic changes associated with cortical neuron degeneration not presynaptic, cholinergic afferents.

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MUSCARINIC (m5) RECEPTORS IN THE VENTRAL TEGMENTAL AREA ARE IMPORTANT FOR BRAIN-STIMULATION REWARD.

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m5 muscarinic receptors are associated with ventral tegmental area (VTA) dopamine cells believed important in reward functions. Muscarinic receptor blockers in VTA raise thresholds for brain-stimulation reward by over 100%. Antisense oligodeoxynucleotides targetted at m5 mRNA were infused into the VTA for 6 days by osmotic minipumps. The m5 antisense oligo, but not m5 sense or m5 missense, raised frequency thresholds by a mean of 62% on days 4-6 in 10 rats. Thresholds returned to baseline within 1-2 days after the infusion stopped. Although oligo infusions caused some damage near the tip in several sites, the selective m5 antisense effects were still strong when damage was minimal. The m5 antisense was more effective as the first infusion than as the second infusion. The density of QNB-labelled muscarinic receptors near substantia nigra and VTA was reduced by m5 antisense infusion in one rat, but D1 and D2 dopamine receptors labelled by emonapride and SCH23990 were largely unaffected. Therefore, m5 muscarinic receptors appear to be important in the mediation of lateral hypothalamic brain-stimulation reward in rats. We are now measuring the effects of m5 antisense infusion in VTA on the density of m1-m5 receptors near VTA measured using selective antibodies (obtained courtesy of A. Levey). Western blots on membrane fractions obtained from ventral midbrain tissue including VTA shows a clear band at about 70 kD with the m5 antibody.

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REDUCED SCOPOLAMINE-EVOKED RELEASE OF ACh IN THE NEOCORTEX OF AWAKE, FREELY-MOVING RATS FOLLOWING TRAUMATIC BRAIN INJURY.

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Several lines of evidence indicate that experimental traumatic brain injury (TBI) can produce spatial memory deficits that are associated with cholinergic hippocampal deficits. However, recent data suggest that the cholinergic cortical system may be more critical to spatial memory performance than the hippocampal cholinergic system (Berger-Sweeney, et al., *J. Neurosci.*, 14(7):4507-4519, 1994). Furthermore, the presence of ACh in the neocortex is reportedly essential for recovery of spatial memory following basal forebrain damage (Winkler, et al., *Nature*, 375:484, 1995). This study examined the effects of TBI on chronic cortical cholinergic neurotransmission by employing *in vivo* microdialysis to examine acetylcholine (ACh) release in the cortex of awake, freely-moving rats two weeks after lateral cortical impact (n=8) or sham surgery (n=8). The rats were anesthetized and a microdialysis probe was placed into the cortex 2 mm lateral to bregma. The animal discontinued from anesthesia, placed in a tether and infused with artificial CSF (1.3 µl/min). After a four-hour equilibration period, ACh were measured at 20-min. intervals prior to and after i.p. administration of scopolamine (1 mg/kg), which evokes ACh release by blocking muscarinic autoreceptors. Injured animals had significantly less ($P<0.01$) ACh release following scopolamine (1.98 ± 0.36 pmoles) compared to sham rats (5.16 ± 0.89 pmoles). The data suggest that the nucleus basalis-cortical cholinergic pathway may contribute significantly to post-traumatic spatial memory deficits. Supported by CDC R49CCR-312296, NIH-R01NS33150 and NIH P50NS30318.

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BIODISTRIBUTION AND PET IMAGING OF MUSCARINIC SUBTYPE SELECTIVE 3-(3-(3-FLUOROPROPYLTHIO)-1,2,5-THIADIAZOL-4-YL)-TETRAHYDRO-1-METHYL-PYRIDINE (FP-TZTP) AND R 3-QUINUCLIDINYL S-FLUOROMETHYL BENZILATE (FMeQNB).

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R,S FMeQNB displays a 7 fold selectivity for M2 in vitro using tissue and subtype specific radioligands. In the rat, uptake of R,S-[F-18]FMeQNB is uniform in all brain regions consistent with the M2 subtype. The uptake in brain is blocked by ~50% upon coinjection with 50 nmol of R,S-FMeQNB. The most dramatic displacement and blocking of R,S-FMeQNB is observed in the heart. In monkeys, kinetic analysis of brain ROIs showed moderate extraction with nearly irreversible behavior in cortical and subcortical regions but not in cerebellum. QNB reduced radioactivity in all brain regions. However, the biodistribution will be more sensitive to delivery than changes in receptor concentration. We also evaluated FP-TZTP for muscarinic subtype selectivity. In rats, uptake of [F-18]FP-TZTP is high, peaking at 15 min. and blocked by P-TZTP. Clearance is slower in the medulla and pons. Analysis of metabolites in the rat brain reveal that the parent compound represents >86% of the activity in the brain, but <10% in plasma at 15 min. In rhesus monkey, studies using [F-18]FP-TZTP revealed two lipophilic metabolites. Brain uptake is rapid and the clearance is sustained over 2 h. Administration of P-TZTP at 60 min. results in a rapid displacement of brain activity. Receptor measurements can be obtained from a one-compartment model fit using [F-18]FP-TZTP.

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ROLE OF CEREBRAL ATROPHY IN THE MEASUREMENT OF MUSCARINIC RECEPTOR DENSITY IN PATIENTS WITH ALZHEIMER'S DISEASE.

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The present study was designed to investigate the effect of cerebral atrophy in the assessment of muscarinic receptor density in patients with mild probable Alzheimer's disease (AD) when compared to age-matched controls. [¹²³I]4-iododexetimide (IDEX) has previously been shown to be useful for muscarinic receptor imaging using Single Photon Emission Computerized Tomography. Five patients with AD (mean age of 77.6 ± 2.1 year (mean \pm S.E.M.)) and a Mini Mental State Examination (MMSE) of 18.6 ± 0.8) and 5 healthy controls (76.4 ± 2.7 year; MMSE of 28.2 ± 0.6) were included. All subjects received a brain SPECT (Strichman camera, 64x64 matrix, slice thickness of 10 mm with a center to center distance of 5 mm) 8-9 hours after the intravenous injection of 5 mCi IDEX (specific activity > 6000 Ci/mmol) and axial MRI (Magnetron 63 2P/4000, 256x256 matrix, slice thickness of 5 mm and a center to center distance of 6.5 mm). All subjects were imaged in axial direction. Subsequently, MRI and SPECT images were combined using chamfer matching. Regions of interest (ROI) were drawn on the frontal (F), parietal (P) and temporoparietal (TP) cortex, and on the basal ganglia. Cerebral atrophy was assessed by calculating the percentage of cerebral spinal fluid within a given ROI. Relative binding of IDEX was calculated with the basal ganglia as a reference. Values are expressed as mean \pm S.E.M.

	Controls	AD	p-value
F	0.88 ± 0.02	0.79 ± 0.04	0.08
P	0.99 ± 0.02	0.92 ± 0.03	0.11
TP	1.00 ± 0.02	0.92 ± 0.03	0.06

Using multiple linear regression analysis, the observed differences proved to be independent of cerebral atrophy. The present results demonstrate that measurement of muscarinic receptor density in patients with AD is independent of cerebral atrophy. Accordingly, IDEX may be useful in the diagnosis of AD.

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CLONING AND EXPRESSION OF THE cDNA FOR AN m1-TOXIN

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Mamba venoms contain trace quantities of toxins that bind with high affinity to m1, m2 and m4 muscarinic receptors (Max *et al.*, J Neurosci 13, 4293, 1993; Liang *et al.*, Toxicon, in press; Valentine *et al.*, Neurosci Abstr 22, 1257, 1996). The first toxins to be isolated that affect muscarinic receptors, MT1 and MT2 (Adem *et al.*, BBA 968, 340, 1988), are now known to have nearly equal affinity for m1 and m4 receptors, and MT1 can act as an agonist (Kornisiuk *et al.*, Toxicon 33, 11, 1995). In contrast, m1-toxins bind selectively and pseudoirreversibly to m1 receptors (Max *et al.*, J Neurosci 13, 4293, 1993; Carsi-Gabrenas and Potter, Neurosci Abstr 22, 1256, 1996), m4-toxin binds reversibly with m4 > > m1 (102-fold) affinity, and both the m1-toxins and m4-toxin are antagonists (Max *et al.*, Neurosci Abstr 19, 462, 1993; Liang *et al.*, Toxicon, in press). At concentrations that fully block m1 receptors, m1-toxins have no blocking activity on m2-m5 receptors. We have cloned and expressed the cDNA for one of our m1-toxins for five reasons; (1) the toxin selected (see below) has the highest specificity for m1 receptors of any of the anti-muscarinic toxins, (2) to obtain much larger amounts of an m1-toxin (grams) than can be obtained by purification (15-180 µg/g dry venom), (3) to permit studies by site-directed mutagenesis of the amino acid residues that confer high toxin affinity and specificity, (4) to permit studies of the amino acid residues that confer antagonist vs. agonist activity, and (5) to facilitate radiolabeling. Messenger RNA was obtained from the venom glands of a green mamba (*Dendroaspis angusticeps*). Degenerate primers to regions of the desired protein sequence were made, and RT-PCR was used to prepare cDNA products. RACE techniques were then used to isolate the full length cDNA for this m1-toxin. This cDNA firmly establishes the mature toxin sequence: LTCVKSNSI-WFPTSEDCPDGQNLFCFKRWQYISPRMYDFTRGCAATCPKAEYRDVINCCGTDKCNK. This cDNA is being used to produce m1-toxin in stable cell lines. [Supported by AG-12976]

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NEW MUSCARINIC TOXINS FROM *D. VIRIDIS* VENOM

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There are more than ten different small proteins from the venom of *Dendroaspis* snakes, which are selective ligands for muscarinic receptor (MACHR) subtypes. Most have been isolated from *D. angusticeps* (Adem *et al.*, 1988; Jerusalinsky *et al.*, 1992; Karlsson *et al.*, 1991) and *D. polylepis* (Karlsson *et al.*, 1994; Jerusalinsky *et al.*, 1994). We have isolated new toxins from *D. viridis* venom using classical chromatography techniques and named them as DvMT. Four fractions out of six, inhibited ³H-NMS binding to rat cerebral cortex membranes. DvMT4 showed selectivity for cloned m4 human MACHR. From inhibition curves of ³H-NMS binding, it was shown that its affinity for m4 receptor is about 100 folds greater than for m1 subtypes, and there was minor or no inhibition to the m2, m3 or m5 subtypes. We also performed inhibition curves in rat cerebral cortical membranes and brain slices. The data from each region have been fitted to competitive inhibition curves, and the distribution of putative m4 receptors in the rat brain have been shown. These experiments were compared with similar assays with the MT1 and 2 from *D. angusticeps*, which show high affinity for m1 and m4 MACHR (MT1: K_i=60 nM and 130 nM; MT2: K_i=420 nM and 1200 nM, for m1 and m4, respectively). From the analysis of the images it has been possible to compare the differential distribution and concentration of MACHR subtypes. We expect that additional new muscarinic toxins with different selectivity, represent an addition to the library of ligands available for studying different subtypes of MACHR.

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MUSCARINIC TOXINS WITH SELECTIVITY FOR MUSCARINIC RECEPTOR SUBTYPES: DIFFERENTIAL BINDING PATTERNS IN CLONED AND NATIVE RECEPTORS.

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MT1, 2 (Adem *et al.*, 1988) and 3 (Jolkkonen *et al.*, 1994) (same as m4-toxin; Max *et al.*, 1993) have been isolated from *Dendroaspis angusticeps* venom. From radioligand binding experiments with relatively selective antagonists, it was suggested that MT1 and 2 bound selectively to m1 central muscarinic receptor (Jerusalinsky *et al.*, 1992). The K_i s for MT1 and MT2, in synaptosomal membranes from cerebral cortex were range from 20-100 nM. In assays with cloned human muscarinic receptors, it was shown that both toxins have high affinity for m1 and slightly lower for m4 receptor. While MT1 and 2 have very little affinity for m5 and do not bind to m2 or m3, MT3 exhibited the highest affinity for m4 ($K_i=2$ nM) with 40-fold lower affinity for m1 ($K_i=78$ nM) and very little affinity for the others (Jolkkonen *et al.*, 1994). The toxins were tested by binding experiments in other native membranes from rat. High affinity binding was found in the hippocampus as well as in the striatum, rich in m4 subtype. There was no interaction with pancreas membranes, rich in m3, nor with atria, rich in m2. Unexpectedly, MT1 and 2 were able to displace ³H-prazosin (α -adrenoceptor antagonist) with a $K_i=0.3$ μ M in cerebral cortex. However, this binding was reversible while appears irreversible to MACHR. MT3 also bound to α -adrenoceptor, but with significant lower affinity. The toxins have been labelled by using 3H-acetic anhydride. The autoradiograms of brain slices showed a special binding pattern for each toxin. The muscarinic toxins, with their high selectivity, high potency and slow reversibility or irreversibility seem to be exceptionally useful tools for studying muscarinic receptor subtypes.

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DIFFERENTIAL EFFECT OF MUSCARINIC TOXIN 3 (MT3) ON BRAIN MUSCARINIC RECEPTORS COUPLED TO ADENYLYL CYCLASE.

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In rat striatum, activation of muscarinic receptors by acetylcholine (ACh) inhibits the adenylyl cyclase (a.c.) activity stimulated by either forskolin (FSK) or dopamine D1 receptor agonists. The muscarinic toxin 3 (MT3), a selective ligand for the m4 muscarinic receptor subtype, antagonizes the ACh inhibitory effects with pA_2 values of 8.10–8.15. The potency is close to the affinity of the toxin for the cloned m4 muscarinic receptor (pK_i 8.7). The MT3 antagonism appears to be competitive and reversible. This finding supports the classification of the striatal inhibitory receptors as M_4 . In rat olfactory bulb, ACh stimulates basal a.c. activity and increases the enzyme activation by Gs-coupled neurotransmitter receptors. On the basis of the sensitivity to a number of classical muscarinic receptor antagonists, the pharmacological profile of this stimulatory response results quite similar to that displayed by the muscarinic inhibition of striatal a.c. ($r = 0.934$). However, only 20–25 % of the ACh stimulation of basal a.c. is antagonized by MT3 with a pK_i of 8.33, with the remaining effect being insensitive to 2 μ M MT3. Also in rat frontal cortex, the muscarinic inhibition of FSK-stimulated a.c. displays a drug sensitivity similar to that of the striatal muscarinic inhibition. However, this response was antagonized by MT3 only at concentrations higher than 1 μ M. Thus, MT3 reveals pharmacological differences not recognized by classical receptor antagonists among muscarinic receptors regulating cyclic AMP formation in rat brain.

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USE OF MUSCARINIC TOXINS, MTx1, MTx2 AND MTx3, IN THE STUDY OF SYNAPTIC TRANSMISSION IN THE PERIPHERAL NERVOUS SYSTEM.

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MTx1 and MTx2 from the Eastern green mamba, *Dendroaspis angusticeps*, have high affinity for m1 receptors compared to their affinity at other subtypes of muscarinic acetylcholine receptors, while MTx3 has high affinity for m4 receptors (1, 2).

We have used the toxins to study the muscarinic receptors in the rabbit vas deferens and the guinea pig uterine preparations. Previously, it was reported, in the rabbit vas deferens preparation, that MTx1 and MTx2 decreased responses to neural stimulation, indicating a presynaptic M₁ acetylcholine receptor controlling release of the transmitters ATP and noradrenaline (3). However, there have been suggestions that this presynaptic muscarinic acetylcholine receptor is of the M₄ subtype. The muscarinic receptor subtype in the guinea pig uterine preparation has been classified by some as M₄ (4) and by others as M₂ (5). To resolve these questions we have used the M₄ specific MTx3.

In the rabbit vas deferens preparation, MTx3 (5-25nM, i.e. up to ten times the K_i concentration at m4 receptors) had no effect on responses to neural stimulation. In contrast, MTx1 and MTx2 (>50nM, i.e. approximately equal to K_i value at m1 receptors) caused a significant decrease in twitches. We confirm that the presynaptic muscarinic acetylcholine receptor in this preparation is M₁. In the guinea pig uterine preparation, MTx3 at 100nM did not antagonize the contractions caused by methacholine. 4-DAMP at 10nM and methoctramine at 100nM did antagonize the responses to methacholine (pK_b values equal 8.95 and 7.7 respectively). We conclude that the muscarinic acetylcholine receptor in this preparation is M₂.

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FINAL REPORT

U.S. Army Medical Research Acquisition Activity

Grant No. DAMD 17-97-1-7004

The recently completed Seventh Symposium on Subtypes of Muscarinic Receptors was judged an outstanding success, upholding the standards for excellence set by the six previous symposia. This judgment was based on: 1) the high quality and timeliness of the invited scientific papers; 2) the large number of first rate and informative posters presented; 3) the invited participation of women and minorities in the scientific sessions; 4) the large number of predoctoral fellows and young scientists whose attendance was made possible by funds granted to the Symposium, and 5) the short oral presentations of selected poster papers.

There were 180 attendees at the Seventh Symposium, half of whom were from 17 countries outside the United States. Twenty-five (25) major papers were presented in the regular sessions, five of these by women scientists. The workshop on The Use of Muscarinic Toxins in the Study of Muscarinic Receptors featured five papers, one by a woman scientist. A total of 91 poster papers were discussed in the two scheduled afternoon sessions, but posters were available for viewing for the entire 4-day meeting. Ten posters were selected for 10 minute oral presentations at an evening session set aside for this new feature. Among the highlights of the subjects presented in the regular sessions were: the role of muscarinic receptor activation in prostate cancer; preclinical and clinical data on tolterodine, a new bladder selective muscarinic antagonist, and clinical data on darifenacin in the treatment of urinary incontinence and functional bowel disease.

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The papers submitted for the Otto Loewi Award were judged by the Committee as good scientific research but not of the quality anticipated for this distinguished award. However, two of the young scientists submitting papers for this award accepted our invitation to attend the Symposium and to present their research in both the poster and oral sessions. It is also noteworthy that three of the papers selected for oral presentations on the basis of their importance and impact were those of predoctoral fellows, and the choice was made by the Committee in the absence of any prior knowledge of the status of the authors.

The Proceedings of the Seventh Symposium, which contains the edited manuscripts of the papers presented in the regular sessions as well as the abstracts of the poster papers, has already been published--only 3 months after the close of the Symposium. The Proceedings appears as Vol.60, Nos 13/14, of the February 21, 1997 issue of Life Sciences and is sent free of charge to the thousands of subscribers of Life Sciences worldwide as well as to hundreds of others including all of our pre- and postdoctoral fellows. Grant support from the U.S. Army Research and Acquisition Activity was largely responsible for the publication of the Proceedings and has been acknowledged in the program distributed to all registrants and in Life Sciences.

We are extremely pleased that the grants we received and the funds contributed by 27 pharmaceutical companies made it possible for us to pay the travel and accommodation expenses of the 25 speakers and committee members from academia. The figure of which we are most proud, however, concerns the large number of pre- and postdoctoral students whom we were able to bring to the Symposium. There were 22 in all and 16 of these young investigators used this opportunity to present the results of their own research. The attendance of these young scientists was made possible and was largely supported by grants from the National Institute of Neurological Disorders

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and Stroke and from the National Science Foundation as well as by contributions from industry. As a direct result of these grants, many of the pre- and postdoctoral fellows were able to attend the annual meeting of the Society for Neuroscience in Washington, D.C. (our venue and dates were purposely chosen with this in mind). A list of the pre- and postdoctoral fellows and their affiliation is appended along with sample copies of the many letters received indicating the benefits derived from, and the overall success of, the Symposium.

Another measure of success of the Symposium was the high degree of active participation of the registrants in the formal sessions of the Symposium and their interaction in informal gatherings. The attendance at each of the five lecture sessions was excellent and appeared to include all registrants. The 5-10 minute period of discussion between papers was informative and provocative. The excellent lighting and space available for posters encouraged lengthy discussions by both presenters and viewers. There appeared to be much enthusiasm for the research being presented particularly by the young scientists. All in all, it is evident that the Seventh Symposium on Subtypes of Muscarinic Receptors has not only encouraged the research efforts of young investigators but has also stimulated and is stimulating additional research which should, indeed, lead to the development of new and better therapeutic agents as well as agents useful to the U.S. Army Research and Acquisition Activity and to the U.S. Army Medical Research Institute Chemical Defense.

Plans for the Eighth Symposium have already been set in motion. The Symposium will be held in Boston in the fall of 1998 in conjunction with the 150th-anniversary of the founding of the first medical school for women in the world which became Boston University School of Medicine 125 years ago. We hope that USAMRAA will again help to support the publication of the Symposium's Proceedings.

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